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AUXOTROPHY AND HETEROTROPHY IN MARINE LITTORAL DIATOMS[1]

JOYCE C. LEWIN AND RALPH A. LEWIN

Abstract

Forty-four pure cultures of marine littoral diatoms (43 pennate and 1 centric) have been studied to determine (*a*) whether any accessory organic factors (vitamins) are essential for growth in light, and (*b*) whether the cells can grow in darkness using glucose, acetate, or lactate as substrates.

Six isolates were shown to require thiamine as sole growth factor; eleven require cobalamin only; and three require both thiamine and cobalamin. Sixteen are capable of heterotrophic growth using glucose but not acetate or lactate; eight can use glucose or lactate; one uses glucose or acetate; one uses glucose, acetate, or lactate; and two use only lactate.

By the classical criteria of valve structure, the clones fall into about 25 different species, belonging to nine genera. However, it is evident that light microscopy is inadequate for the specific descriptions of many small diatoms. Electron micrographs of acid-cleaned frustules of each isolate served as an aid to specific identifications. Nutritional studies have demonstrated the existence of a range of distinct physiological races within the "species" *Amphora coffaeiformis*, *Nitzschia closterium*, and *N. frustulum*.

Introduction

The density of phytoplankton in the ocean has generally been observed to increase around the littoral margins, where the shallowness of the water and the propinquity of land provide conditions more favorable for plant growth than in the open ocean. This littoral flora consists largely of diatoms. These comprise not only centric species, which for the most part are truly planktonic, but also an abundance of pennate forms, usually associated with growth on solid substrata. Although the brown, slimy layer of diatoms on sand, mud, and attached algae in shallow waters and rock pools is a familiar feature of the shore, this littoral flora is taxonomically less well known than the diatoms of the oceanic plankton. As an example of our lack of knowledge of these pennate forms, Hustedt (13) described 89 new species out of a total of 369 species identified in two mud samples sent to him from Beaufort, North Carolina. The richness of this flora must be attributable at least in part to the variety of organic and inorganic substances derived from the nearby land and from decaying seaweeds and other organic matter in the mud.

[1]Manuscript received August 12, 1959.

Contribution No. 1062 from the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts. These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the Woods Hole Oceanographic Institution, NR-104-392.

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As a preliminary contribution to our understanding of the ecology of marine littoral diatoms, a study of the physiology of such forms is needed. The present investigation deals with the requirement of certain species for accessory organic growth factors, namely vitamins (i.e. auxotrophy), and with their ability to use glucose, acetate, or lactate for growth in darkness (i.e. heterotrophy). The range of species was limited to tolerant forms capable of growing well under the empirical conditions of laboratory culture. This accounts largely for the recurrence of similar species from different localities.

Materials and Methods

Unless otherwise indicated, the diatoms were collected and isolated free from bacterial contaminants by R.A.L. Many of the strains were isolated from crude selective cultures in sea water enriched with mineral supplements, as given below. Most were purified by streaking on enriched sea water agar; a few were isolated by micromanipulation.

Specific identification of the species was facilitated by the use of electron micrographs of cleaned frustules. However, additional complications were introduced thereby, since many fine structural details, revealed by electron microscopy but not perceptible otherwise, could not have been taken into consideration in the original descriptions of the species. Moreover, the examination of clonal cultures has provided considerable information on intraspecific variation, not hitherto available to taxonomists working with field collections. In spite of our conviction that many diatom species are inadequately described, we have endeavored to assign a binomial to each isolate.[2] The following works were consulted for this purpose: Boyer (1), Cholnoky (5), Cleve-Euler (6), Helmcke and Krieger (11), Hustedt (12, 13), Peragallo and Peragallo (19), Schmidt (21), Van Heurck (22).

Stock cultures were maintained in 125-ml Erlenmeyer flasks containing 50 ml of filtered sea water enriched with 0.1 g/l. $Ca(NO_3)_2.4H_2O$; 0.02 g/l. K_2HPO_4; 0.05 g/l. $Na_2SiO_3.9H_2O$; 0.5 mg/l. Fe; 0.3 mg/l. Zn; 0.1 mg/l. each of B, Co, Cu, Mn, Mo; 1 μg per liter of vitamin B_{12}; and 1.0 g/l. Tryptone (Difco). Stock cultures were also maintained in test tubes on 1.0% agar slants of the same medium.

For determination of the vitamin requirements of these diatoms, sea water was first treated with decolorizing carbon, Norit A (Pfanstiehl), at a concentration of 1 g per 100 ml for $\frac{1}{2}$–1 hour at room temperature, and then filtered. This treatment removed cobalamin (2, 17) and other vitamins originally present. To determine whether cobalamin was required, the growth of each diatom was tested in Norit-treated sea water (50 ml in a 125-ml Erlenmeyer flask) with and without cobalamin addition, but otherwise enriched as above. It was found that Tryptone, a rich source of most organic nutrilites including thiamine, supplies no detectable cobalamin to the medium. For the

[2]Cultures of the diatoms used in this investigation have been deposited in the Algal Culture Collection, Botany Department, Indiana University, Bloomington, Indiana. Permanent slide mounts of the cleaned silica walls have been deposited at the Smithsonian Institution, Washington, D. C.

determination of other vitamin requirements, Tryptone was omitted, and growth was tested with and without the addition of a vitamin mixture containing 1 mg each of thiamine, niacin, and biotin per liter. Essential vitamins were identified by the use of single supplements. A vitamin was evidently required in those cases where growth in the absence of a nutrilite was negligible, or where a short period of growth was followed after a few days by bleaching of the cells. In the presence of the required vitamins, the cells were capable of sustained growth, and bleaching did not occur. No attempt was made to reveal additional vitamin requirements by a second transfer, since carry-over with the first inoculum was considered negligible. Inoculum was transferred with a bacteriological loop; in the case of vitamin B_{12}, it was estimated that vitamin contamination by carry-over did not exceed 10^{-4}–10^{-5} μg per liter.

For determination of heterotrophic growth, each species was tested in light and in darkness on agar medium: (*a*) as in the preceding paragraph, i.e., control without carbon source, (*b*) with 0.5% glucose, autoclaved separately, (*c*) with 0.1% Na acetate, and (*d*) with 0.2% Na lactate. Heterotrophic growth was confirmed in liquid media.

Results

In Table I are presented data on the following points: (1) vitamins, if any, found to be essential for autotrophic growth in light, using carbon dioxide as the sole major carbon source; and (2) the ability of the strain to grow heterotrophically in darkness, using glucose, acetate, or lactate as sole carbon source, in the presence of requisite vitamins. Since in many cases the specific determination was in some doubt, information is also given on the size range observed in our cultures and on the number of striae per unit length. These data, together with electron micrographs of cleaned frustules (Figs. 1–30), serve further to identify the species in case of question.

The two groups of diatom strains classified as *Nitzschia frustulum* (Kütz.) Grun. (6 isolates) and *Amphora coffaeiformis* Ag. (10 isolates) exhibited considerable intraclonal variation, and intergradation between clones did not permit clear separation into recognized varieties. Evidently each of these species comprises several physiological races (see Table I). One isolate classified as *Nitzschia closterium* W. Sm. (No. 54) differed considerably from the other three strains of this species both in cell size and in physiological characteristics, although the fine structure of the silica walls appeared identical.

The numbers of isolates exhibiting requirements for vitamins and ability to grow heterotrophically are summarized in Table II. All diatoms capable of heterotrophic growth were pigmented when grown in the dark. No correlation between auxotrophy and facultative heterotrophy was observed.

Discussion

The diatoms reported on in this paper were isolated from inshore littoral habitats and probably include no planktonic species. Since centric diatoms generally do not grow well on agar, only pennate forms were obtained by the

TABLE I

Auxotrophy and heterotrophy in various diatom strains

Isolate No.	Fig. No.	Name	Size of valve, μ	Striae in 10 μ	Origin	Requires vitamin:		Grows in dark on:		
						B_1	B_{12}	Gl.	Ac.	La.
—	1	*Achnanthes brevipes* (Ag.) Cl.	40–50×9–11	16	Great Pond, Mass. (from S. Watson, No. 7)	−	+	−	−	−
40	7	*Amphipleura rutilans* (Trentepohl) Cl.	11–16×4	24	Herring Cove, N.S. (rock pool)	+	+	−	−	−
73	30	*Amphiprora paludosa* W. Sm. var. *duplex* (Donk.) Cl.	25×10	36	Woods Hole, Mass. (tank)	+	−	−	−	−
34	11	*Amphora coffaeiformis* Ag.	12–22×4	27	Lehave Islands, N.S. (brackish ditch)	−	−	+	−	−
45	—	*Amphora coffaeiformis* Ag.	6–14×4	33	Halifax, N.S. (inlet)	−	+	−	−	−
47	—	*Amphora coffaeiformis* Ag.	12–18×4	30	Woods Hole, Mass. (brackish pond)	−	+	+	−	+
51	9	*Amphora coffaeiformis* Ag.	25×5	26	San Diego, Cal. (mud)	−	−	+	−	−
56	—	*Amphora coffaeiformis* Ag.	4–10×2.5	—	La Jolla, Cal. (tidal rock)	+	+	+	−	−
64	—	*Amphora coffaeiformis* Ag.	5–6×2	27	San Francisco, Cal. (saline pool)	−	−	+	−	−
—	—	*Amphora coffaeiformis* Ag.	13–18×5	32	Sapelo Is., Georgia (shore) (from T. Starr, D 2)	+	−	+	−	−
—	—	*Amphora coffaeiformis* Ag.	13–19×4	24	Galveston, Texas (from T. Starr, D 106)	+	−	+	+	−
—	—	*Amphora coffaeiformis* Ag.	8–13×4	27	Beaufort, N.C. (from T. Rice)	−	−	+	−	−
—	10	*Amphora coffaeiformis* Ag.	6–13×4	30	Rockaway Beach, N.Y. (sea water) (from L. Provasoli)	−	+	−	−	+
59	8	*Amphora lineolata* Ehr.	30–45×10–18	23	Pacific Beach, Cal. (mud)	−	+	−	−	−
44	3	*Navicula meniscullus* Schum.	12–17×6–7	20	Eagle Pt., N.S. (mud)	−	−	−	−	−
78	6	*Navicula* sp.	5–6×2.5	20–25	Sandwich, Mass. (marine canal)	−	−	−	−	−
66	2	*Navicula incerta* Grun.	8–16×5–6	14	San Francisco, Cal. (saline pool)	−	−	+	−	−
69	—	*Navicula incerta* Grun.	12–16×5	16	Salton Sea, Cal. (shore)	−	−	+	−	−
35	24	*Nitzschia angularis* var. *affinis* (Grun.) Perag.	30×3.5	67	Herring Cove, N.S. (on *Chondrus*)	−	−	+	−	−

36	28	*N. filiformis* W. Sm.	60×6	36	Lehave Islands, N.S. (brackish ditch)	−	−	+	−	−
8	—	*N. frustulum* (Kütz.) Grun.	6×3	34	New Haven, Conn. (shore)	−	−	−	−	−
13	17	*N. frustulum* (Kütz.) Grun.	7–16×3	36–41	Prospect, N.S. (rock pool)	−	−	+	−	+
39	—	*N. frustulum* (Kütz.) Grun.	5–12×4	28	Lehave Islands, N.S. (brackish ditch)	−	−	−	−	−
53	18	*N. frustulum* (Kütz.) Grun.	7–16×4–5	23–32	La Jolla, Cal. (tidal rock)	−	+	+	−	−
58	19	*N. frustulum* (Kütz.) Grun.	5–12×4	41	San Diego, Cal. (tidal mud)	−	+	−	−	−
68	—	*N. frustulum* (Kütz.) Grun.	6–28×5	22–24	Salton Sea, Cal. (water sample)	−	−	+	−	−
71	14	*N. hybridaeformis* Hust.	44×8	32	Woods Hole, Mass. (tank)	−	−	−	−	−
72	23	*N. laevis* Hust.	11–18×5	52	Woods Hole, Mass. (tank)	−	−	+	−	+
—	—	*N. laevis* Hust.	8–11×3.5	57	(from S. Watson, No. 5)	−	−	+	−	+
5	15	*N. curvilineata* Hust.	38×9	39	New Haven, Conn. (shore)	−	−	−	−	+
—	12	*N. marginata* Hust.	27–67×1.5–2	54	(from S. Watson, No. 10)	−	−	+	−	−
63	16	*N. obtusa* W. Sm. var. *scalpelliformis* Grun.	75×9	27–31	San Diego, Cal. (shore)	−	−	−	−	−
49	22	*N. ovalis* Arnott	8–10×3–4	52–66	Vineyard Sound, Mass. (water sample)	−	+	−	−	−
—	21	*Nitzschia* sp. (cf. *N. ovalis* Arnott)	8–12×5–7	44	(from S. Watson, No. 18)	−	−	−	−	−
52	27	*N. punctata* (W. Sm.) Grun.	10×7	20	San Diego, Cal. (tidal mud)	−	+	+	−	−
—	20	*N. tenuissima* Perag.	9–33×3	50–55	Beaufort, N.C. (from T. Rice)	?	?	+	+	+
—	13	*Nitzschia* sp.	12–17×2	52	(from S. Watson, No. 12)	−	?	−	−	−
—	—	*N. closterium* (Ehr.) W. Sm.	26–60×7	—	Galveston, Texas (from T. Starr, D 105)	+	−	+	−	+
—	—	*N. closterium* (Ehr.) W. Sm.	45–60×7	—	Sapelo Is., Georgia (from T. Starr)	+	−	+	−	+
—	26	*N. closterium* (Ehr.) W. Sm.	40–45×7	—	Beaufort, N. C. (from T. Rice)	+	−	+	−	+
54	25	*N. closterium* (Ehr.) W. Sm.	18–36×7	—	San Diego, Cal. (mud)	+	+	−	−	−
27	5	*Synedra affinis* Kütz.	50×6	12	Herring Cove, N.S. (rock pool)	−	+	−	−	−
11	4	*Stauroneis amphoroides* Grun.	13–30×5	27	Herring Cove, N.S. (rock pool)	−	−	−	−	−
—	29	*Cyclotella* sp.	4–18	—	Martha's Vineyard, Mass. (from R. Guillard)	−	+	+	−	−

TABLE II

Numbers of isolates exhibiting auxotrophy or heterotrophy

Vitamins required	No. of isolates	Growth in dark on:	No. of isolates
Thiamine only	6	Glucose only	16
Cobalamin only	11	Glucose or lactate	8
Thiamine + cobalamine	3	Glucose or acetate	1
None	24	Glucose, acetate, or lactate	1
		Lactate only	2
Total	44	No growth in dark on any	16
		Total	44

ordinary bacteriological techniques employed, and only one centric species was included in this study.

It should be pointed out that the range of physiological features among the species investigated here is not necessarily representative of the littoral flora, because diatoms with certain growth-factor requirements may have been eliminated by the methods of isolation. The proportion of vitamin-requiring species among those examined here is certainly an underestimate of that in nature. The fact that about a third of the strains required cobalamin reflects the abundance of this vitamin in marine muds (3, 4); in inshore waters it may rarely fall below limiting values (9). There have apparently been no comparable studies on the free thiamine in sea water, but since nine diatom strains were found to require this vitamin for growth, thiamine, too, may often be in adequate supply in littoral sea water. The absence of strains requiring other vitamins, such as biotin and niacin, may be a consequence of inadequate supplies of these substances in nature and in the media employed for preliminary isolations. Vitamin requirements by diatoms, in all cases marine, have been previously reported by Hutner and Provasoli (14), Droop (7, 8, 10), and Lewin (18). (It is our present opinion that the organism referred to as "*A. perpusilla*" by Hutner and Provasoli (14) should be designated *A. coffaeiformis*, and that "*Fragilaria brevistriata*" of Lewin (18) should be *Synedra affinis* Kütz. These organisms have been listed by their preferred names in Table I.)

Several species of fresh-water diatoms are capable of heterotrophic growth using glucose (15). Among 60 carbon sources tested, glucose was the only one which supported growth of *Navicula pelliculosa* in darkness (15). In order to limit the range of substrates to be tested here for heterotrophic growth, a representative sugar and two acids were selected. Glucose and acetate were chosen, since virtually all algae shown to be capable of heterotrophy can utilize at least one of these substrates. In addition, lactate was tested since it is actively respired by *N. pelliculosa*, although it does not support heterotrophic growth (15, 16). Since three of the marine diatoms studied here (strains 13, 36, 53) were capable of digesting agar, as indicated by the formation of pits at the site of individual colonies, it appeared possible that galactose might also support growth in darkness. However, in a preliminary test with the 15 species then available, including strains 5, 8, 11, 13, 27, and others not

included in Table I, none was able to use galactose for growth. The fact that approximately half of the isolates were able to grow heterotrophically is of some ecological significance, indicating a means of nutrition other than photosynthesis if suitable carbon sources are available. Plunkett (20) has demonstrated free sugars (sucrose, glucose, fructose, galactose, arabinose, xylose) in deep-sea sediments; presumably littoral muds would be even richer in such compounds.

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A great deal of difficulty was encountered in the identification of some of the small diatoms employed in this study. Dr. Paul S. Conger (Smithsonian Institution, Washington, D.C.) spent considerable time and effort in checking some of our tentative identifications and in certain cases suggested more appropriate designations, for which we are very grateful.

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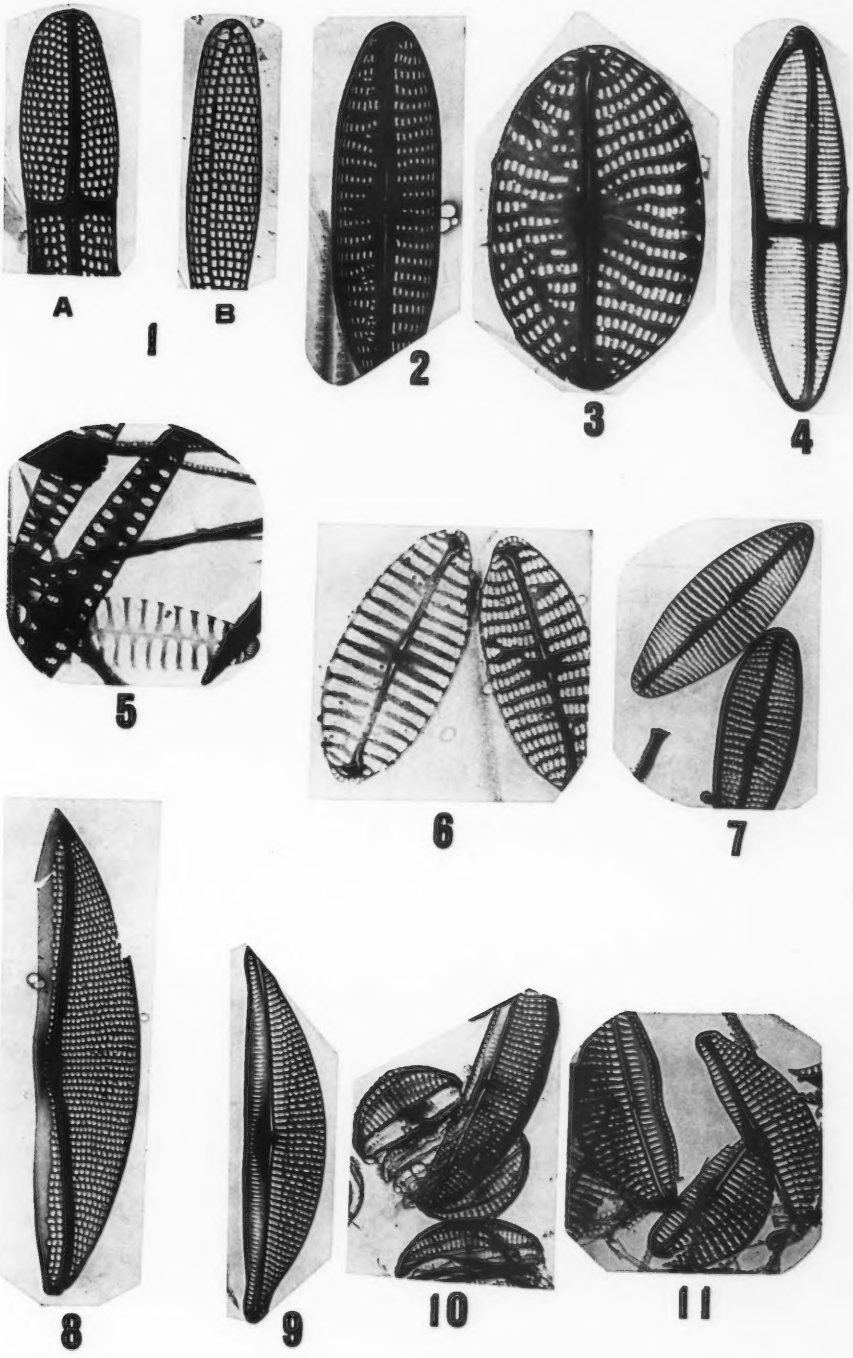
EXPLANATION OF FIGURES

FIG. 1. *Achnanthes brevipes* (Ag.) Cl. ×1200
FIG. 2. *Navicula incerta* Grun. ×3850
FIG. 3. *Navicula menisculus* Schum. ×5610
FIG. 4. *Stauroneis amphoroides* Grun. ×2600
FIG. 5. *Synedra affinis* Kütz. ×3000
FIG. 6. *Navicula* sp. ×5610
FIG. 7. *Amphipleura rutilans* (Trentepohl) Cl. ×3000
FIG. 8. *Amphora lineolata* Ehr. ×2400
FIG. 9. *Amphora coffaeiformis* Cl. ×2400
FIG. 10. *Amphora coffaeiformis* Cl. ×2400
FIG. 11. *Amphora coffaeiformis* Cl. ×3000
FIG. 12. *Nitzschia marginata* Hust. ×7200
FIG. 13. *Nitzschia* sp. ×4800
FIG. 14. *Nitzschia hybridaeformis* Hust. ×2400
FIG. 15. *Nitzschia curvilineata* Hust. ×3000
FIG. 16. *Nitzschia obtusa* W. Sm. var *scalpelliformis* Grun. A, ×1200; B, ×7720
FIG. 17. *Nitzschia frustulum* (Kütz.) Grun. ×3000
FIG. 18. *Nitzschia frustulum* (Kütz.) Grun. ×3850
FIG. 19. *Nitzschia frustulum* (Kütz.) Grun. ×3850
FIG. 20. *Nitzschia tenuissima* Perag. ×7200
FIG. 21. *Nitzschia* sp. (cf. *N. ovalis* Arnott) ×5610
FIG. 22. *Nitzschia ovalis* Arnott ×5610
FIG. 23. *Nitzschia laevis* Hust. ×3850
FIG. 24. *Nitzschia angularis* var. *affinis* (Grun.) Perag. ×6000
FIG. 25. *Nitzschia closterium* (Ehr.) W. Sm. ×3600
FIG. 26. *Nitzschia closterium* (Ehr.) W. Sm. ×3850
FIG. 27. *Nitzschia punctata* (W. Sm.) Grun. ×5610
FIG. 28. *Nitzschia filiformis* W. Sm. ×3000
FIG. 29. *Cyclotella* sp. A, ×5610; B, ×3850
FIG. 30. *Amphiprora paludosa* W. Sm. var. *duplex* (Donk.) Cl. ×5610

NOTE: Figs. 1–30 follow.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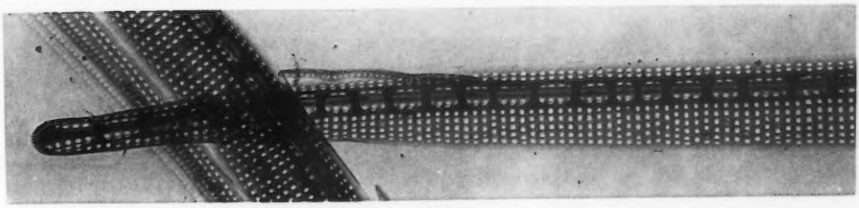

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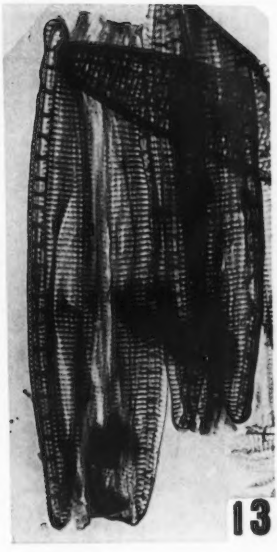

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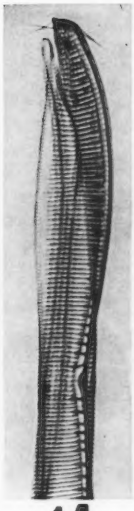

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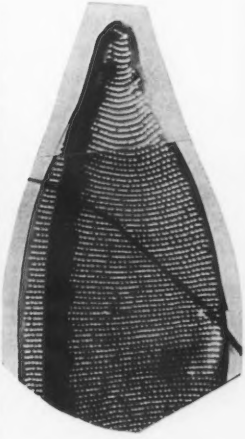

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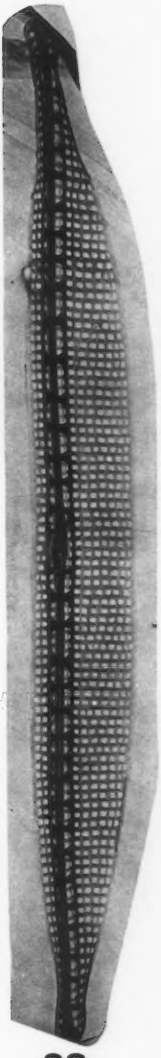

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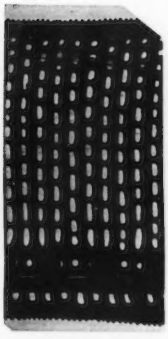
A



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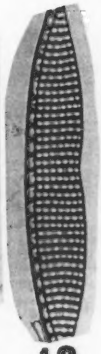

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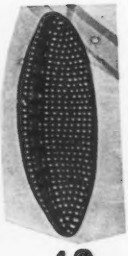

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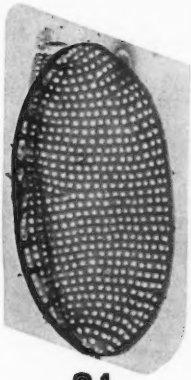

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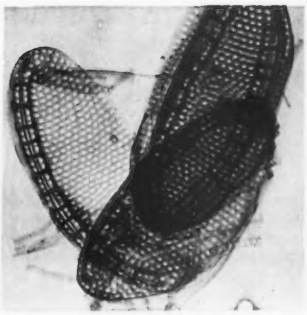

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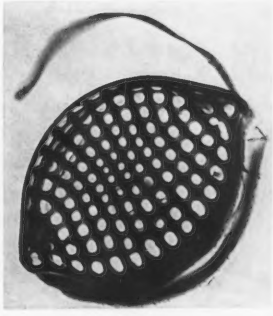

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A

B

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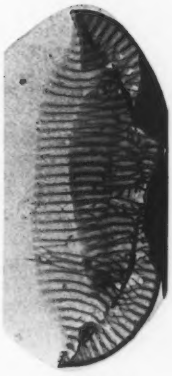

30

ISOLATION AND INVESTIGATION OF INDUCED ASPOROGENIC MUTANTS[1]

D. G. LUNDGREN AND G. BESKID[2]

Abstract

A simple chemically defined (minimal) medium was developed which supported good growth and sporulation of *B. cereus* var. *lacticola*. Normal growth and sporulation habits in the medium were determined employing an active growth-culture technique. Induced asporogenic mutants were isolated employing ultraviolet light as the mutagen. Mutants differed in their sporulation habits from the normal culture when grown at 37° C under the standardized conditions in a minimal synthetic medium. Three mutant cultures appeared to be temperature-sensitive mutants as regards sporulation, that is, in the minimal medium mutant cultures were able to sporulate when grown at 28° C but were non-sporulating at 37° C. Sporulation blocks were partially reversed when casein hydrolyzate and some low molecular weight protein building blocks were added to the synthetic medium. The pH changes of the medium during growth and sporulation of normal and mutant cultures were compared.

Introduction

We now know considerable about bacterial sporulation because of the recent biochemical approach to render intelligible the sporulation mechanism. In support of this statement reference can be made to recent review articles (3, 4, 16), the collection of papers on *Spores* (5), and a special issue of the Journal of Applied Bacteriology (8).

Advancement in our understanding of the mechanism of sporulation has come from the application of new experimental techniques which have clearly shown that biochemical processes occurring during sporulation are multiphasic and different from those common to vegetative growth. Today, it is possible to separate the processes of sporulation from those related to vegetative growth permitting a detailed investigation of sporulation using physiologically homogeneous populations.

The authors have applied active growth-culture techniques (2, 6, 11) and radiation-induced asporogenic mutants for attacking a sporulation problem. This report describes the production of non-sporulating mutants and the screening for chemical agents capable of supporting sporulation in mutant cells which are asporogenic when grown in the minimal synthetic medium. Further, some preliminary studies are reported on the effects of temperature on asporogenesis as well as pH changes which occur in the minimal medium during culture development of normal and mutant cells. An abstract of this work has been reported (9).

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Contribution from the Department of Bacteriology and Botany, Syracuse University, Syracuse, New York. Part of a thesis submitted in partial fulfillment for the degree of Doctor of Philosophy. This investigation was supported by a research contract from the United States Atomic Energy Commission.
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Experimental Methods

Cultural Procedures

Investigations were made with *Bacillus cereus* var. *lacticola* (A.T.C.C. No. 4342). A minimal synthetic medium was developed which supported good growth and sporulation. Initially, the common glucose – glutamic acid – salts medium of Hardwick and Foster (7) was tried, but results showed that a modification was required for maximum growth and sporulation. Changes included the addition of glycine and $ZnSO_4$ as well as doubling the glutamate concentration and decreasing the amount of glucose by one-half. The new minimal medium (GGGS medium) consisted of: glucose, 5 g; glutamic acid, 10 g; glycine, 0.1 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; $FeSO_4.7H_2O$, 0.01 g; $MnSO_4.4H_2O$, 0.01 g; $ZnSO_4.7H_2O$, 0.013 g; $CaHPO_4$, 2 ml (saturated solution); distilled water, to 1 l. The pH of the medium after autoclaving was 6.7. Whenever a solid medium was required 15 g of agar per liter were added. Normal sporulation patterns were established for *B. cereus* grown in 250-ml Erlenmeyer flasks containing 50 ml of GGGS medium on a reciprocal shaker at both 28° C and 37° C. One milliliter of a suspension of washed *B. cereus* spores was used as an inoculum. The spore inoculum was prepared from 7-day-old cultures grown at 37° C in Kolle flasks containing a 1% yeast extract – agar medium. After incubation, spores were washed from the agar surface with a salt solution identical with that of the synthetic medium except Zn was absent. The spores were centrifuged, separated from the liquid wash, and resuspended in a fresh salt solution; this washing procedure was repeated six times. The final turbidity of the washed spore suspension was adjusted to give an opacity of 355 Klett units, heat-shocked, and stored in the refrigerator. Spores remained physiologically active for over 30 days when treated as described. A spore crop was never used as an inoculum for longer than one month. Cell inocula of each of four asporogenic mutants were prepared by growing vegetative cells in Kolle flasks at 37° C on the GGGS-agar medium for 24 hours prior to harvesting. The turbidity of the washed cell suspensions was adjusted to give a reading of 355 Klett units. A 1-ml inoculum was used routinely. Mutant cell crops were not used for investigations after 1 month's storage, even though all mutant vegetative cell crops remained physiologically active for well over 30 days. Normal sporulation habits were also determined for cultures of *B. cereus* grown on a solid GGGS medium; hundreds of clones were examined to make certain that these normal sporulation patterns also were predictable. The percentage of spore-bearing cells was determined in all instances by microscopic counts of heat-fixed films stained with malachite green and safranin.

Radiation-Induced Mutants

A procedure similar to that used for the induction of nutritional mutants of *B. subtilis* by Burkholder and Giles (1) was employed for the induction and isolation of biochemical mutants. A 5-ml suspension of washed spores in sterile petri plates was exposed to the mutagen, in this instance ultraviolet light. The light source was a Hanovia utility model quartz lamp with an

energy output, under the conditions of the experiments, of 2060 microwatts/cm². Half of the energy came from a wave length of 2537 Å. All suspensions were rotated during irradiation by means of a constant-speed turntable (Nuclear-Chicago model PM-1). After irradiation, a 0.5-ml portion of appropriate dilutions of the spore suspensions was spread over the dry surface of the GGGS recovery medium with a bent glass rod. Recovery plates for each dilution were made in quadruplicate, storing two plates at 28° C and two plates at 37° C; plates were incubated for 5 days. Bacterial clones developing from spores surviving irradiation necessary to accomplish at least a 65% kill were screened for asporogenic mutants. A microscopic examination of stained heat-fixed smears was the only mutant screening procedure employed. The surviving clones were carefully compared with those clones developing from normal non-irradiated cells. Mutant clones (consisting of all vegetative cells) were coded and transplants to agar slants were made for future reference.

Chemical Screening

Known metabolites, and complex sources of chemicals, were screened, as regards their ability to promote sporulation in four asporogenic variants. The spectrum of chemicals screened was extensive enough to cover most known nutrilites, growth factors, and metabolites. Investigations were carried out in shake culture at 37° C in 50 ml of GGGS medium supplemented with the chemical addition(s). However, with asporogenic culture No. 107 (also a nutritional mutant) alanine, valine, leucine, and isoleucine at 100 p.p.m. (each) were added to the GGGS medium to support vegetative growth paralleling that of the normal *B. cereus*. The pH of the media was adjusted to 6.8 just prior to inoculation. One milliliter of the test inocula was added to each flask. The various chemical additions investigated are listed in Table I. The "B" vitamin mixture tested included (concentration expressed as mg/50 ml medium): riboflavin, 0.025; thiamin.HCl, 0.00005; *p*-aminobenzoic acid, 0.005; nicotinic acid, 0.005; calcium pantothenate, 0.005; pyridoxine.HCl, 0.005; folic acid, 0.0005; choline chloride, 0.10; *i*-inositol, 0.050; biotin, 0.00005; vitamin B_{12}, 0.00005. The components of the mixture comprising the Krebs cycle intermediates were (concentrations were 100 p.p.m. each): sodium pyruvate, sodium succinate, *cis*-aconitate, alpha-ketoglutaric acid, citric acid, DL-malate, and sodium fumarate. The mixture containing the nucleic acid components consisted of (concentration expressed as mg/50 ml medium): thymine, 5.0; cytosine, 0.78; uracil, 5.0; adenine, 5.0; guanine, 5.0; xanthine, 5.0; hypoxanthine, 5.0; uridine, 5.0; adenylic acid, 5.0; guanylic acid, 5.0; adenosine triphosphate, 5.0. The amino acid solution listed in Table I consisted of seven amino acids; these were: diaminopimelic acid, lysine, methionine, threonine, isoleucine, DL-homoserine, and aspartic acid. All amino acids of this solution and those listed in Table I not designated by a configuration were L amino acids; D and DL amino acids are listed accordingly. The vegetative cell wall peptide components (listed in Table I as VCWPC) and the spore wall peptide components (listed in Table I as SWPC) were those

TABLE I

Effects of various chemical additions on growth and sporulation of four asporogenic mutants in a minimal synthetic medium

Chemical additions	Concn.	Strain							
		40	52	54	107	40	52	54	107
		Klett units				% spores			
Casein hydrolyzate[a]	200 p.p.m.	730	730	730	–	12	22	16	–
Casein hydrolyzate	500 p.p.m.	730	730	730	–	28	40	33	–
Casein hydrolyzate	1000 p.p.m.	690	700	660	720	48	59	44	0
Casein hydrolyzate	1600 p.p.m.	740	730	730	–	22	25	26	–
Casein hydrolyzate	2000 p.p.m.	650	650	650	–	0	0	0	–
Alanine, valine, and isoleucine	100 p.p.m. each	–	700	–	–	–	2	–	–
Serine and threonine	100 p.p.m. each	–	720	–	–	–	0	–	–
Cysteine, cystine, and methionine	100 p.p.m. each	–	690	–	–	–	0	–	–
Aspartic acid	100 p.p.m.	–	720	–	–	–	9	–	–
Phenylalanine and tyrosine	100 p.p.m. each	–	800	–	–	–	0	–	–
Tryptophan, hydroxyproline, histidine, and proline	100 p.p.m. each	–	900	–	–	–	0	–	–
Aspartic acid	300 p.p.m.	–	690	–	–	–	0	–	–
Lysine	300 p.p.m.	–	630	–	–	–	0	–	–
Arginine	300 p.p.m.	–	710	–	–	–	6	–	–
Lysine and arginine	100 p.p.m. each	–	630	–	–	–	24	–	–
Lysine and arginine	200 p.p.m. each	–	630	–	–	–	22	–	–
Lysine and arginine	300 p.p.m. each	–	630	–	–	–	18	–	–
DL-Citrulline, proline, and arginine	100 p.p.m. each	750	690	700	700	0	10	0	0
Amino acid solution	100 p.p.m. each	435	540	360	640	0	0	0	0
Serine and cysteine	100 p.p.m. each	680	660	680	640	0	0	0	0
Alanine, valine, and leucine	100 p.p.m. each	610	570	580	–	7	0	0	–
Tryptophan, phenylalanine, tyrosine, and histidine	100 p.p.m. each	720	670	720	700	0	0	0	0
DL-Cystathionine	100 p.p.m.	680	680	680	650	0	0	0	0
DL-Lanthionine	100 p.p.m.	710	710	710	710	0	0	0	0
DL-α-Amino-*n*-butyric acid	100 p.p.m.	600	600	600	660	10	0	0	0
2,4-Diaminobutyric acid	100 p.p.m.	700	700	700	660	0	0	0	0
Ornithine	100 p.p.m.	700	700	650	650	0	8	6	0
VCWPC	100 p.p.m. each	620	620	620	730	11	9	8	0
SWPC	100 p.p.m. each	700	670	670	730	24	24	12	0
Glutathione	100 p.p.m.	660	710	710	660	0	0	0	0
L-Glutamine	200 p.p.m.	750	760	750	710	5	7	2	0
L-Glutamine	600 p.p.m.	–	720	–	–	–	4	–	–
L-Asparagine	200 p.p.m.	750	730	750	710	4	5	9	0
L-Asparagine	600 p.p.m.	720	670	720	–	1	17	3	–
Glutamic acid and glycine[b]	200 p.p.m. each	720	670	670	730	0	0	0	0
Glutamic acid and glycine[c]	200 p.p.m. each	720	670	670	730	0	3	0	0
Glycyl-DL-alanine	100 p.p.m.	710	710	690	670	0	0	0	0
Glycyl-DL-α-amino-*n*-butyric acid	100 p.p.m.	670	670	670	670	0	0	0	0
Glycyl-L-asparagine	100 p.p.m.	730	730	700	720	0	0	0	0
DL-Alanyl-DL-asparagine	100 p.p.m.	730	730	660	690	0	3	5	0
L-Leucylglycine	100 p.p.m.	620	620	620	680	0	6	0	0
D-Leucylglycylglycine	10 p.p.m.	720	–	–	–	12	–	–	–
D-Leucylglycylglycine	50 p.p.m.	700	–	–	–	6	–	–	–
D-Leucylglycylglycine	100 p.p.m.	690	690	690	600	28	8	6	0
D-Leucylglycylglycine	200 p.p.m.	700	–	–	–	14	–	–	–
DL-Leucylglycylglycine	100 p.p.m.	660	660	–	–	16	33	–	–
DL-Leucylglycylglycine	150 p.p.m.	700	700	–	–	12	5	–	–
DL-Leucylglycylglycine	200 p.p.m.	700	670	–	–	2	3	–	–
Glycylglycylglycylglycine	100 p.p.m.	790	790	700	680	0	2	0	0
Collagen hydrolyzate	100 p.p.m.	650	630	630	680	0	0	2	0
Phytone	100 p.p.m.	690	650	650	690	0	0	7	0
Lactalysate	100 p.p.m.	670	670	620	560	0	6	10	0

TABLE I (*Continued*)

Effects of various chemical additions on growth and sporulation of four asporogenic mutants in a minimal synthetic medium (*Continued*)

Chemical additions	Concn.	Strain							
		40	52	54	107	40	52	54	107
		Klett units				% spores			
Proteose peptone	400 p.p.m.	740	700	700	800	0	0	0	0
Carboxypeptidase[d]	100 p.p.m.	700	570	700	660	0	0	11	0
DPN[d]	60 p.p.m.	710	670	690	610	17	6	4	0
Protease[d]	100 p.p.m.	700	670	750	700	0	0	0	0
Pepsin[d]	100 p.p.m.	750	640	660	670	0	2	0	0
Diaminopimelic acid	100 p.p.m.	700	700	700	620	0	0	0	0
Dipicolinic acid	100 p.p.m.	650	630	570	570	0	0	0	0
Dipicolinic acid	300 p.p.m.	0	0	0	0	0	0	0	0
Yeast extract	200 p.p.m.	750	720	750	730	0	4	0	0
Yeast extract	1000 p.p.m.	770	730	730	720	8	14	16	0
Yeast extract	2000 p.p.m.	–	750	750	–	0	14	16	–
Yeast extract	4000 p.p.m.	–	550	550	–	–	0	0	–
Beef extract	60 p.p.m.	800	750	750	670	0	1	1	0
Beef extract	120 p.p.m.	750	670	670	670	0	1	0	0
Hydrolyzed DNA	167 p.p.m.	680	700	670	670	0	0	0	0
Hydrolyzed DNA	333 p.p.m.	700	700	700	680	0	4	0	0
Hydrolyzed RNA	167 p.p.m.	670	670	650	620	0	9	0	0
Hydrolyzed RNA	333 p.p.m.	670	680	650	650	0	10	0	0
Nucleic acid components	1.0 ml	690	750	620	510	0	0	0	0
Krebs cycle intermediates	0.5 ml	760	760	720	405	0	7	0	0
Krebs cycle intermediates	1.0 ml	760	720	720	450	0	0	0	0
"B" vitamin mixture	0.3 ml	700	650	680	580	2	2	0	0
12-hr veg. cell hydrolyzate	0.5 ml	690	680	670	445	0	0	0	0
12-hr veg. cell hydrolyzate	1.0 ml	690	700	670	545	0	0	0	0
18-hr veg. cell hydrolyzate	0.5 ml	700	700	690	325	0	1	0	0
18-hr veg. cell hydrolyzate	1.0 ml	700	700	700	475	0	2	3	0
29-hr spore hydrolyzate	0.5 ml	700	710	700	500	0	0	0	0
29-hr spore hydrolyzate	1.0 ml	700	710	700	620	0	0	0	0
Glucose	500 p.p.m.	630	630	630	630	0	0	2	0
D (+) Galactose	100 p.p.m.	710	710	660	630	0	8	0	0
L (+) Rhamnose	100 p.p.m.	720	650	660	690	0	4	0	0
Fructose-1,6-diphosphate	100 p.p.m.	710	650	660	690	1	5	0	0
Glucosaminic acid	200 p.p.m.	750	700	700	700	6	10	3	0
Glucosaminic acid	600 p.p.m.	–	720	–	–	–	9	–	–
D (+) Glucosamine	200 p.p.m.	730	720	690	750	11	7	6	0
D (+) Glucosamine	600 p.p.m.	–	700	–	–	–	0	–	–
Cholesterol	100 p.p.m.	350	430	415	375	0	0	0	0
Dehydrocholesterol	100 p.p.m.	670	690	690	710	0	0	0	0
Soya sterols	100 p.p.m.	430	495	440	305	0	0	0	0
Estradiol	100 p.p.m.	650	690	690	700	0	0	0	0
Capric acid (com. grade)	0.1 ml	183	216	200	189	0	0	0	0
n-Caproic acid (com. grade)	0.1 ml	0	0	0	0	0	0	0	0
Oleic acid (com. grade)	0.1 ml	560	405	500	370	0	0	0	0
Alpha-picolinic acid	100 p.p.m.	680	700	690	430	0	0	0	0
Ethylenedinitrilotetraacetic acid	100 p.p.m.	580	580	580	290	0	0	0	0
Salt solution I	0.1 ml	700	700	700	790	8	4	0	0
Salt solution II	1.0 ml	700	660	680	760	16	12	8	0
$ZnSO_4.7H_2O$	13 p.p.m.	670	710	670	–	0	5	0	–
$ZnSO_4.7H_2O$	39 p.p.m.	680	670	620	–	0	5	0	–
$FeSO_4.(NH_4)_2SO_4.6H_2O$	28 p.p.m.	730	680	680	–	0	8	5	–
$FeSO_4.(NH_4)_2SO_4.6H_2O$	84 p.p.m.	720	670	670	–	0	4	0	–
$CoSO_4.7H_2O$	0.02 p.p.m.	246	520	315	–	0	0	0	–
$CoSO_4.7H_2O$	0.06 p.p.m.	0	0	0	–	0	0	0	–
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.006 p.p.m.	730	660	660	–	0	3	5	–
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.018 p.p.m.	680	620	620	–	0	0	0	–
$CuSO_4.5H_2O$	0.04 p.p.m.	780	650	650	–	0	4	2	–

TABLE I (*Concluded*)

Effects of various chemical additions on growth and sporulation of four asporogenic mutants in a minimal synthetic medium (*Concluded*)

Chemical additions	Concn.	Strain							
		40	52	54	107	40	52	54	107
		Klett units				% spores			
$CuSO_4.5H_2O$	0.12 p.p.m.	710	650	650	–	0	9	5	–
$NaHCO_3$	67 p.p.m.	750	740	700	560	0	0	0	0
Alpha-ketoglutaric acid	117 p.p.m.	700	750	620	440	0	0	0	0
$NaHCO_3$ and alpha-ketoglutaric acid	67 p.p.m. 117 p.p.m.	700	730	700	520	0	1	0	0
Casein hydrolyzate[a] and tryptophan	2000 p.p.m. 10 p.p.m.	700	670	670	710	0	0	3	0
Casein hydrolyzate[a] and tryptophan	6000 p.p.m. 100 p.p.m.	630	700	630	730	0	0	0	0
Casein hydrolyzate,[a] lysine, and arginine	1000 p.p.m. 100 p.p.m. each	–	700	–	–	–	59	–	–
Casein hydrolyzate[a] and SWPC	1000 p.p.m. 100 p.p.m. each	670	670	650	650	33	34	21	0
Casein hydrolyzate[a] and L-asparagine	1000 p.p.m. 600 p.p.m.	–	720	–	–	–	41	–	–
Casein hydrolyzate[a] and D-leucylglycylglycine	1000 p.p.m. 100 p.p.m.	710	710	710	750	52	37	56	0
Casein hydrolyzate[a] and DL-leucylglycylglycine	1000 p.p.m. 100 p.p.m.	710	710	710	750	48	44	49	0
Casein hydrolyzate[a] and DL-leucylglycylglycine	1000 p.p.m. 150 p.p.m.	700	700	700	–	37	37	18	–
Casein hydrolyzate[a] and L-leucylglycylglycine	1000 p.p.m. 100 p.p.m.	710	710	710	750	47	53	42	0
Casein hydrolyzate[a] and DPN	1000 p.p.m. 60 p.p.m.	710	–	–	–	49	–	–	–
Casein hydrolyzate[a] and yeast extract	1000 p.p.m. 100 p.p.m.	680	680	660	720	4	0	0	0
Casein hydrolyzate[a] and hydrolyzed DNA	1000 p.p.m. 333 p.p.m.	700	700	700	750	9	36	14	0
Casein hydrolyzate[a] and hydrolyzed RNA	1000 p.p.m. 333 p.p.m.	740	740	680	770	0	10	8	0
Casein hydrolyzate,[a] hydrolyzed DNA, and hydrolyzed RNA	1000 p.p.m. 333 p.p.m. 333 p.p.m.	700	700	700	700	0	1	0	0
Casein hydrolyzate[a] and DNA	1000 p.p.m. 100 p.p.m.	700	700	670	770	43	45	48	0
Casein hydrolyzate[a] and RNA	1000 p.p.m. 100 p.p.m.	630	660	660	700	37	53	41	0
Casein hydrolyzate[a] and DNA and RNA	1000 p.p.m. 100 p.p.m. each	660	660	640	720	29	56	47	0
Casein hydrolyzate[a] and nucleic acid components	1000 p.p.m. 1.0 ml	740	740	740	680	0	0	0	0

[a]Acid-hydrolyzed vitamin-free casein, General Biochemicals Inc., Chagrin Falls, Ohio. The hydrolyzate was adjusted to a pH of 6.8 and autoclaved separately.
[b]Added at the time of inoculation.
[c]Added after 16 hours' incubation.
[d]Enzyme preparations added after 10 hours' incubation:
DPN (Diphosphopyridine nucleotide)—Sigma Chemical Co., St. Louis, Missouri.
Carboxypeptidase—Mann Research Laboratories, New York, N.Y.
Protease—Mann Research Laboratories, New York, N.Y.
Pepsin—Mann Research Laboratories, New York, N.Y.

reported by Powell and Strange (14). The former included: alanine, aspartic acid, lysine, glutamic acid, and glucosaminic acid, while the latter contained: alanine, aspartic acid, diaminopimelic acid, glutamic acid, and glucosaminic acid. Salt solution I consisted of: $ZnSO_4.7H_2O$, 1.3; $FeSO_4.(NH_4)_2SO_4.6H_2O$,

2.8; $CoSO_4.7H_2O$, 0.002; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.0006; $CuSO_4.5H_2O$, 0.004; concentrations expressed as p.p.m. based upon the weight of the whole molecule. Salt solution II contained the same components as salt solution I, but in different concentrations; these were 13, 28, 0.02, 0.006, and 0.04 p.p.m., respectively. Cell hydrolyzates were prepared by heating sealed tubes containing 500 mg (wet weight) of either vegetative cells or spores suspended in 5 ml of 6 *N* HCl, at 121° C for 1 hour. DNA and RNA hydrolyzates were prepared in a like manner except that the hydrolyzing vehicle was 6 ml of 3 *N* NaOH. Control tests were run with all chemical screening experiments; specifically, 1 ml of a normal *B. cereus* spore suspension was inoculated into 50 ml of the minimal glucose – glutamic acid – glycine – salts medium. Over 200 control tests were run with normal *B. cereus*.

Temperature Investigations

Growth and sporulation in four non-sporulating isolates were studied at 28° C. All studies employed shake culture in glucose – glutamic acid – glycine – salts medium with a 54-hour incubation period. Inoculation procedures were the same as those reported for growth and sporulation studies at 37° C. Quantitation of growth and sporulation was by turbidity and stained smears respectively.

pH Investigations

Normal and asporogenic cultures were grown in a series of 250-ml Erlenmeyer shaken flasks containing 50 ml of glucose – glutamic acid – glycine – salts medium at both 28° C and 37° C. Each flask receiving the normal culture (*B. cereus* spores) and each receiving the mutant cultures (vegetative cells) was inoculated following the previously described procedures. At different time intervals, flasks were removed from the shaker and pH determinations of the medium performed. The pH of undiluted cultures was determined with a Beckman pH meter using a glass–calomel electrode system.

Results

Growth and Sporulation Patterns of Normal B. cereus

The minimal GGGS medium supported growth and sporulation of the parent strain of *B. cereus*. In shake culture, the spore ⟶ vegetative cell ⟶ spore cycle was complete by 31 hours and over 90% sporulation occurred. The first signs of sporulation, as observed in heat-fixed films, appeared after 17 hours. At this time, a small non-refractile dark-green staining area was observed in most members of the population. By 20 hours, well-developed spores appeared which stained a light green; at the 30th hour, sporangia began to break releasing the spores. Growth and sporulation curves are shown in Fig. 1 for normal *B. cereus* cells grown in shake flasks in the minimal medium at both 28 and 37° C. The growth and sporulation pictures were remarkably consistent.

Results showed that zinc was required for sporulation; it had no appreciable effect upon growth. Both glycine and glutamic acid were required for growth

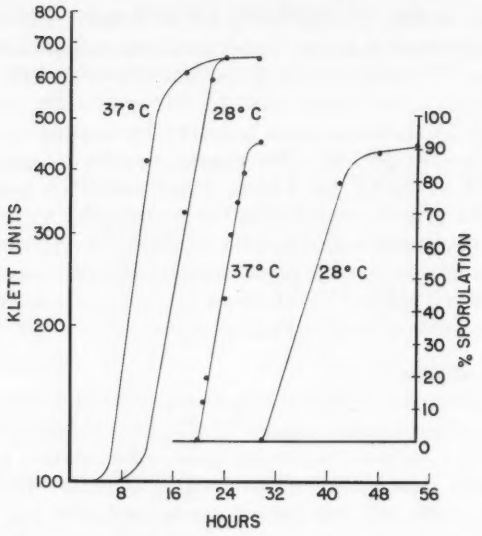

FIG. 1. Growth and sporulation curves for normal *B. cereus* cells grown in a synthetic minimal medium. Percentage sporulation values represent cells possessing fully formed refractile spores.

and spore formation. No appreciable turbidity developed when glutamic acid was omitted from the medium. The addition of inorganic nitrogen supplies, such as $NaNO_3$ (10 g/liter) and $(NH_4)_2SO_4$ (10 g/liter) in place of the two amino acids failed to promote growth of *B. cereus*.

Radiation-Induced Asporogenic Mutants

Normal *B. cereus* clones when cultured on the solid synthetic medium consistently showed 95–100% spores after 5 days' incubation at either 28 or 37° C. Clones developing from irradiated survivors and showing some type of metabolic lesion(s) interfering with the sporulation processes were free of spores. To date, 918 clones developing from irradiated survivors have been examined, and of these, 19 clones were found to be vegetative only. All 19 clones were isolated from plates incubated at 37° C. These preliminary results indicate a mutant frequency of 2.1%. The immediate objective of the irradiation work was to secure non-sporulating mutants; consequently the authors were not directly concerned with studying mutation rates. Further, they realize the smallness of the sampling number from which the mutant frequency was derived. The value 2.1% is merely given as a likely order of magnitude. Of the 19 asporogenic cultures isolated, four were selected for investigation; these were mutant strains of *B. cereus* identified as Nos. 40, 52, 54, and 107. Culture No. 107 demonstrated a partial nutritional deficiency as expressed by a long lag period when compared with the normal culture. Growth patterns for culture No. 107 paralleled that of the parent when leucine, isoleucine, valine, and alanine were added to the minimal medium. No sporulation

occurred in culture No. 107 when grown in the minimal medium either with or without the four amino acids; the culture did produce a pink water-soluble pigment during late stages of cultural growth. Mutants Nos. 40, 52, and 54 demonstrated vegetative growth characteristics similar to those of the parent culture when cultured at 37° C in the glucose – glutamic acid – glycine – salts medium. No sporulation occurred in these variants at this temperature. Growth patterns for the four variants when cultured under standard conditions at 37° C are shown in Fig. 2.

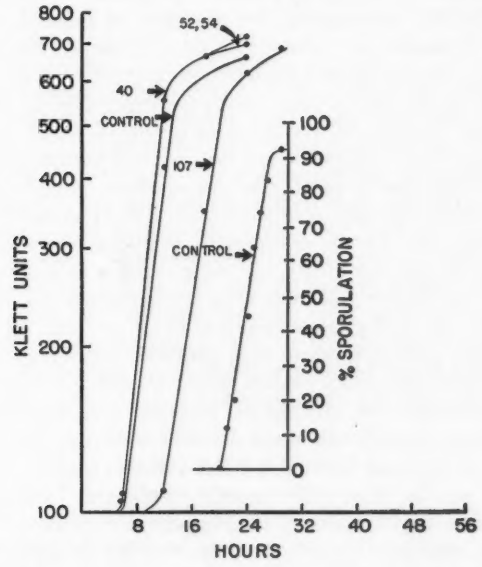

FIG. 2. Growth and sporulation curves for mutant and normal *B. cereus* cells cultured at 37° C in a synthetic minimal medium. Percentage sporulation values represent cells possessing fully formed refractile spores.

hemical Screening

The purpose of the screening tests was to find specific chemical compound(s) which when added to the minimal (glucose – glutamic acid – glycine – salts) medium would support spore formation in the mutant cultures which were always non-sporulating in the minimal medium at 37° C. Results of screening tests are shown in Table I. Final Klett readings (24 hours) are shown to indicate the growth relationships between the organism grown in GGGS medium (Fig. 2) and in minimal medium plus the chemical addition(s). Percentage sporulation was determined after 31 hours which was the established time for the completion of the spore ⟶ vegetative cell ⟶ spore cycle. Normal *B. cereus* cultures, tested as a control, and grown in the minimal medium without any addition always gave essentially the same growth and sporulation picture as shown in Fig. 1. The (–) sign shown in the table means no test was

carried out with that particular strain. Of the 134 different chemical additions investigated none showed a complete reversal of the asporogenic condition in any of the four mutant cultures. Maximum spore formation of approximately 50% was noted in cultures Nos. 40, 52, and 54 when casein hydrolyzate (1000 p.p.m.) was added to the minimal medium. The percentage sporulation fell with lower and greater concentrations of casein hydrolyzate. No sporulation has been observed in mutant culture No. 107 in the presence of any of the tested supplements. In culture No. 52, lysine and arginine when added together supported approximately 20% sporulation; when added alone neither supported significant sporulation. Asparagine, the B-amide of aspartic acid, partially supported spore formation in culture No. 52. The amide was more effective than aspartic acid. The spore wall peptide components (alanine, aspartic acid, diaminopimelic acid, glutamic acid, and glucosaminic acid) were somewhat effective for strains Nos. 40, 52, and 54 to an extent of 24, 24, and 12% spores respectively, whereas the vegetative cell wall peptide components when added to the minimal medium had no effect upon sporulation. None of the components of either of these peptides used singly was effective in supporting sporulation in the mutant cultures. Of the commercial peptides investigated, only the D and DL forms of leucylglycylglycine were capable of partially supporting spore formation. The D form was more effective in culture No. 40 (28% spores) while the DL form showed greater response in culture No. 52 (33% spores). When amino acids, peptides, spore wall peptide components, and complex organic supplements were each added singly to the GGGS medium along with casein hydrolyzate, the sporulation response was no greater than that observed with casein hydrolyzate alone. In some instances, i.e. in the presence of yeast extract, hydrolyzed DNA and RNA and tryptophan, spore formation was significantly smaller than with casein hydrolyzate. DPN was one of the supplements giving slight sporulation support for culture No. 40, while carboxypeptidase additions to the medium resulted in 11% sporulation in culture No. 54. The two organic acids, diaminopimelic acid and dipicolinic acid, had no effect upon sporulation. Complex additions such as yeast extract showed very slight sporulation support for organisms Nos. 52 and 54; beef extract had no effect upon sporulation. Additions of hydrolyzed nucleic acids, mixture containing the Krebs cycle intermediates, "B" vitamin mixture, hydrolyzates of washed spores and vegetative cells, sterols, fatty acids, and single element additions had no effect upon sporulation. Some sporulation support was noted when salt solution II was added to the minimal GGGS medium. In most cases, the various additions to the minimal medium had little effect upon total growth of the four mutant strains. This can be seen from a comparison of the Klett readings shown in Table I with the growth picture as shown in Fig. 2 and might be considered as indirect evidence in support of the feeling that the additions were available to the cells at the time of normal sporulation. Further, when additions were added at the 16th hour (footnote *c*, Table I) no major differences in growth and/or sporulation was observed. At the 24-hour period all cultures were in the early stationary phase and if the cultures were to form spores, sporulation was under way.

No significant changes in turbidity readings were noted from the 24th hour to the 31st hour. Chemical additions that completely inhibited growth of the four test cultures were *n*-caproic acid, dipicolinic acid (300 p.p.m.), and $CoSO_4 . 7H_2O$ (.06 p.p.m.). Partial growth inhibition was found with cholesterol, soya sterols, capric acid, oleic acid, and ethylenedinitrilotetraacetic acid. Some supplements partially inhibited growth of culture No. 107 only. These were: lactalysate, alpha-picolinic acid, nucleic acid components, Krebs cycle intermediates mixture, "B" vitamin mixture, all vegetative cell hydrolyzates, 0.5-ml addition of the spore hydrolyzate, $NaHCO_3$, and alpha-ketoglutaric acid.

Temperature Investigations

Two observations during the research led to the investigation of sporulation in the mutant isolates at 28° C; these were: (1) all non-sporulating mutants were isolated at 37° C, (2) normal *B. cereus* cultures sporulated at 28° C. Figure 3 shows the growth and sporulation curves for mutant and normal cultures when grown in the minimal medium in shaken flasks at 28° C. Mutant cultures Nos. 40, 52, and 54 apparently were not asporogenic in the minimal medium at 28° C; they were asporogenic when grown at 37° C. All growth curves showed a longer lag period, but maximum growth was attained at approximately the same time as when the cells were incubated at 37° C. Sporulation at 28° C appeared to be similar for all cultures; spore formation commenced around the 28th hour and was completed by the 50th hour. Normal *B. cereus* cells cultured at 37° C completed sporulation by 28 hours. Mutant

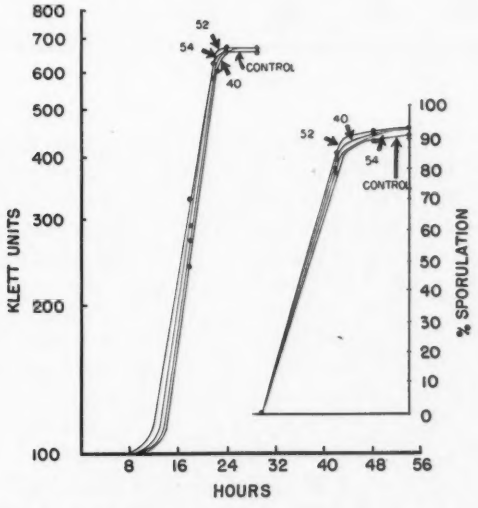

FIG. 3. Growth and sporulation curves for mutant and normal *B. cereus* cells cultured at 28° C in a synthetic minimal medium. Percentage sporulation values represent cells possessing fully formed refractile spores.

culture No. 107 was not investigated, for at 28° C no growth occurred. The stability of temperature sensitivity as related to sporulation in the three mutant cultures was checked by demonstrating that spores produced at 28° C in cultures Nos. 40, 52, and 54 did not form spores (after germination and subsequent vegetative growth) when cultured at 37° C in the minimal synthetic medium. However, when spores produced at 28° C were reinoculated into fresh minimal medium and incubated at 28° C, the mutant spores did go on to complete the spore ⟶ vegetative cell ⟶ spore cycle.

The ability of the mutant cells to form spores as the temperature is raised above 28° C falls off rather rapidly (Table II). Temperature effects on the normal culture were absent but as the temperature was increased, the time for the completion of the spore ⟶ vegetative cell ⟶ spore cycle was shortened.

TABLE II

Effects of temperature on spore formation in mutant cultures of *B. cereus* grown in a minimal synthetic medium

Temperature, °C	Strain							
	40	52	54	Parent	40	52	54	Parent
	Growth							
	Klett units*				% spores*			
28	650	670	670	600	89	86	89	91
30	660	660	660	650	11	30	35	90
32	700	700	700	700	12	7	16	93
34	700	700	700	700	4	6	3	93
37	700	680	680	650	0	0	0	90

*Growth and percentage spore determinations were made at: 54th hour at 28° C and 30° C; 42nd hour at 32°C; 34th hour at 34° C; 31st hour at 37° C.

pH Investigations

Tables III and IV show the pH changes of the medium during growth and sporulation of the normal and four asporogenic *B. cereus* cultures incubated at 28 and 37° C. At 37° C in the normal culture, the medium reached a pH low of 5.9 by the 16th hour at which time the culture was approaching the middle of the logarithmic growth phase. The pH rose steadily from the 16th hour and reached a value of 8.2 by the completion of the spore cycle. Morphological evidence indicated the presence of a prespore at the 16–17th hour. Complete morphogenesis to a mature spore occurred by 30 hours. Although similar growth was observed with the three mutant cultures, both the pH changes and sporulation differed. In the mutant cultures, the pH of the medium dropped after 14 hours' shaking to 5.4, 5.3, and 5.2 for cultures Nos. 40, 52, and 54 respectively. These pH values are lower than that of 5.9 for the normal culture. A steady increase in pH occurred from the 14th hour, reaching a maximum value of 8.3 by 30 hours. These cultures were in approximately the same stage of the logarithmic growth phase as was the normal *B. cereus* culture. Morphological evidence was indicative of some cellular rearrange-

TABLE III

pH changes during the progress of growth and sporulation at 37° C in normal and asporogenic cultures of *B. cereus* grown in a minimal medium

Organism	Time (hr)	Turbidity, Klett units	pH	% spores
B. cereus (normal)	0	0	6.6	100 (inoculum)
	2	7	6.6	0
	12	160	6.2	0
	14	360	6.2	0
	16	425	5.9	0
	20	590	7.6	6
	30	660	8.2	90
B. cereus (mutant 40)	0	0	6.6	0
	2	13	6.6	0
	12	340	5.6	0
	14	485	5.4	0
	16	570	6.8	0
	20	650	7.7	0
	30	730	8.3	0
B. cereus (mutant 52)	0	0	6.6	0
	2	7	6.6	0
	12	345	5.5	0
	14	470	5.3	0
	16	485	6.1	0
	20	650	7.8	0
	30	710	8.3	0
B. cereus (mutant 54)	0	0	6.6	0
	2	7	6.6	0
	12	360	5.7	0
	14	475	5.2	0
	16	570	7.0	0
	20	660	7.8	0
	30	720	8.3	0
B. cereus (mutant 107)	0	0	6.6	0
	2	12	6.6	0
	12	56	5.8	0
	14	230	5.4	0
	16	360	5.7	0
	20	650	7.3	0
	30	730	7.8	0

ments in the mutants by the 14th hour but no normal sporulation ever occurred. Mutant 107 demonstrated a different behavior from that of its sister mutants. By the 14th hour, the pH of the medium had dropped to a low of 5.4; however, the pH rise during subsequent growth was much slower, reaching only 5.9 at the 18th hour compared with values of 7.3 or greater for the other three mutants. The extent of growth of culture No. 107 was equal to that of the other cultures. A high value of 8.1 was recorded at the 28th hour, which dropped to 7.8 at the 30th hour. A pink water-soluble pigment was produced during growth of culture No. 107. When normal and mutant cells were cultured at 28° C, none of the culture media showed as large a pH drop as when the cells were cultured at 37° C. Low pH values for the minimal medium were: parent cells, 6.4; mutant No. 40, 6.1; mutant No. 52, 6.2; mutant No. 54, 6.2. The low pH values of the media appeared earlier in the logarithmic growth phase than was the case at 37° C. Spore formation always commenced at the higher pH values; pH levels of 8.0 for the medium were recorded for the mutant cultures while a value of 7.6 was noted for the control culture medium.

TABLE IV

pH changes during the progress of growth and sporulation at 28° C in normal and asporogenic cultures of *B. cereus* grown in a minimal medium

Organism	Time (hr)	Turbidity, Klett units	pH	% spores
B. cereus (normal)	0	0	6.6	100 (inoculum)
	6	12	6.5	0
	12	25	6.4	0
	18	181	6.5	0
	24	440	7.0	0
	30	660	7.7	1
	40	680	8.1	46
	54	680	8.4	90
B. cereus (mutant 40)	0	0	6.6	0
	6	25	6.5	0
	12	33	6.1	0
	18	320	6.2	0
	24	650	7.5	0
	30	700	8.0	3
	40	730	8.3	74
	54	710	8.4	90
B. cereus (mutant 52)	0	0	6.6	0
	6	20	6.5	0
	12	24	6.3	0
	18	296	6.2	0
	24	620	7.6	0
	30	680	8.0	1
	40	730	8.4	74
	54	720	8.5	91
B. cereus (mutant 54)	0	0	6.6	0
	6	22	6.5	0
	12	28	6.5	0
	18	310	6.2	0
	24	650	7.7	0
	30	700	8.0	5
	40	730	8.4	74
	54	730	8.6	91

Discussion

The development of techniques enabling the study of the sporulation process divorced from that of growth has greatly assisted sporulation research. Hardwick and Foster (3) first used resting cells as a technique of separating growth (reproduction) and spore formation. Sporulation in resting vegetative cells has been referred to as "endotrophic" sporulation. The active growth-culture technique employed in this work also enabled a separation of the growth and sporulation processes. Using as the normal culture inoculum large numbers of washed spores chilled by refrigeration, all the cells in shaken culture developed simultaneously, which resulted in a physiologically homogeneous population. Mature, heat-resistant spores developed after completion of the logarithmic growth phase. Essentially complete sporulation occurred during the few hours which constituted the stationary growth phase. This active-culture technique allows an investigation of cells at any stage of spore formation. Further, in a chemically defined medium, this technique gives the investigator remarkable control of the exogenous chemical environment during the initial spore ⟶ vegetative cell ⟶ spore cycle. Once spores

have formed, which is usually accompanied by mass liberation of vegetative cell contents into the medium as well as germination starting a second growth-spore cycle, chemical control of the medium is no longer possible. With the active growth-culture experiment, essentially endotrophic sporulation is achieved where the cells switch over to a completely endogenous metabolism as they enter the stationary phase.

Biochemical reactions of cells are regulated by genes. Normal cells sporulate, for they possess the necessary functional genes to carry out the responsible biochemical events. Interfering with the orderly reproduction of these genes (i.e., exposure of cells to radiation) may upset the normal organization of biochemical events and thus prevent sporulation, but have no effect upon cell propagation. Such an interference would promote the development of non-sporulating strains, that is, asporogenic mutants. Results of our irradiation studies of sporulating *B. cereus* cells showed that metabolic lesions could be induced in these cells which resulted in sporulation blocks but have no effect upon the growth process. In at least one instance (culture No. 107) the induced lesion(s) did interfere with the normal growth process. Here was a biochemical mutant showing a partial requirement for amino acids in order to maintain a growth response parallel to that of its parent. Before one induces asporogenesis in cells, it is first necessary to establish the normal growth and sporulation requirements, for when cultured in a minimal synthetic medium organisms are required to make full use of their own biochemical machinery for growth and spore formation.

To date, the results of the chemical screening tests employing induced non-sporulating strains have given no direct evidence as to the involvement of specific metabolites, growth factors, etc. in the bacterial sporulation process. A possible clue as to the involvement of amino acids and peptides has come from the work with casein hydrolyzate. However, since casein hydrolyzate contains such a complex of amino acids, keto acids, peptides, etc. it is presently impossible to be specific. Casein hydrolyzate (1000 p.p.m.) neutralized the apparent metabolic lesion(s) interfering with spore formation in three mutant cultures better than any other chemical addition. No single addition (or combinations) of low molecular weight protein building blocks was able to duplicate the response given by casein hydrolyzate. The amino acid combination of lysine and arginine, the spore wall peptide components, and the tripeptide, leucylglycylglycine, gave the best sporulation response. Irradiation of the normal culture has induced mutation(s) that appear to be extremely complex. It should be noted that the mutants have been demonstrated to be extremely stable. The bacterial mutants have been repeatedly cultured at 37° C in the minimal synthetic medium under the established conditions with the complete absence of spores. To date, only 4 of the 19 mutants have been investigated and perhaps some of the other 15 isolations will prove to be more simple as regards genetic changes and their relationship to spore formation.

The aspect of temperature sensitivity as it relates to sporogenesis in the mutant cultures is most interesting. Mutant cultures were non-sporulating in the minimal medium when grown at 37° C. These cultures did undergo

definite morphological changes as witnessed in stained smears. Especially significant was the appearance of very large vacuoles which disappeared at about the time sporulation was expected. However, at this temperature sporulation was always "abortive". Within these same mutant cultures when grown at 28° C under identical culture conditions, sporulation proceeded normally and appeared to be similar to sporulation as found in the parent *B. cereus* at both 28 and 37° C. Maximum sporulation in the three mutant cultures was restricted to temperatures around 28° C. As the temperature increased sporulation was inhibited. At temperatures below 28° C growth did not occur. The phenomenon of temperature sensitivity in *B. cereus* as it relates to sporulation may be compared with the nutritional temperature-sensitive mutants of *Neurospora* (10). A riboflavin mutant was able to grow without riboflavin at 25° C, but at 37° C it would not grow in the vitamin's absence. Presently, a more detailed consideration is being given the sporulation–temperature relationship.

The pH changes in the medium occurring during growth and sporulation of different species of *Bacillus* have been reported in at least two instances (6, 15); these pH changes were similar but different extremes were noted. A semisynthetic medium was employed in the above-cited investigations. Results of the present investigations showed the same general pH picture but with pH changes even more extreme, especially for those changes on the acid side of the pH scale. Certainly, these different pH changes of the medium observed at 28 and 37° C and occurring in cultures displaying similar growth must reflect some differences in the cells' biochemical mechanism. The differences may be only in reaction rates but nevertheless, qualitative differences may exist. Recent work coming from the Laboratories of Bacteriology, University of Illinois (12, 13), has shed some light upon the pH changes. The decrease in pH during growth was attributed to the accumulation of organic acid (pyruvic and acetic) intermediates. And, results of this recent work suggest the absence of a complete oxidative pathway in *B. cereus* T when grown aerobically in glucose. Significance of the pH changes in the minimal medium to sporulation in *B. cereus* mutants must await more explorations.

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THE EXTRACELLULAR POLYSACCHARIDE OF GELATINOUS STRAINS OF CHROMOBACTERIUM VIOLACEUM[1]

WILLIAM A. CORPE

Abstract

A method for the isolation and purification of the voluminous extracellular polysaccharide of *Chromobacterium violaceum* is presented. The purified product was fibrous when wet but dried into a tough, pliable film which was completely water soluble, forming a highly viscous solution. Hydrolysis of the polysaccharide, isolation and identification of the components established the presence of glucose as the principal sugar. A uronic acid and an amino sugar not conclusively identified were also present. Glucose, uronic acid, and the amino sugar were found in an approximate 5:1:1 ratio.

Introduction

Certain strains of *Chromobacterium violaceum* produce tough, gelatinous colonies on nutrient agar and thick, rubbery surface growth on nutrient broth (5). Preliminary examination indicated the rubbery consistency of the growth was due to an extracellular polysaccharide, which besides having a tremendous water-holding capacity and high viscosity in dilute solution was found to be quite resistant to microbial degradation. Soil–polysaccharide enrichment cultures yielded no bacteria or filamentous fungi capable of hydrolyzing the polysaccharide when the latter substance was used as a carbon source in a medium supplemented with ammonia, casamino acids, or peptone as a nitrogen source.

Cultures producing the polysaccharide frequently yielded non-gelatinous mutant colonies on agar media (5), some of which, when isolated, were stable, indicating either loss of ability to accumulate the extracellular polysaccharide at the cell surface or indeed the loss of ability to synthesize the polysaccharide altogether.

The purpose of the present work was to study methods of production, isolation, and purification of the gelatinous polysaccharide and to identify its principal components, using non-gelatinous mutants as controls throughout each operation.

Methods

Cultures

Gelatinous strains of *Chromobacterium violaceum* were used for production of the extracellular polysaccharides. These strains have been described by Corpe (6) and Sneath (20). Strain designations of D_3G, 30, and 32 will be used in the text. D_3G and D_3S were isolated in 1950 and both of these were isolated

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from a parent culture D_3 which after some months in cultivation yielded gelatinous (G) and non-gelatinous (S) variants, that is, extracellular polysaccharide producing and non-producing strains respectively. These were secured by plating and picking until a degree of stability was obtained. The S type culture was used in this study as a control for comparison with the growth and extracellular polysaccharide synthesis by gelatinous cells. Gelatinous strains 30 and 32 are more recent isolates. The cultures were maintained on the following medium: 0.5% peptone, 0.5% yeast extract, 0.1% glucose, and 1.5% agar dissolved in distilled water and the pH was adjusted to 7.0. Transfers were made at 2-week intervals.

Medium for Polysaccharide Production

The organisms were transferred from stock cultures into broth medium of the same composition shown above and incubated at 30° C on a rotary shaker with an excentricity of 3/4 in. and rotating at a speed of 160 r.p.m. The cultures were transferred again, after the appearance of maximum turbidity, into the production medium, which contained 1% glucose, 1% peptone in distilled water with the pH adjusted to 7.0. The vessels used in stationary cultures were liter bottles containing 100 ml of medium. Since isolation of polysaccharide from cultures in shake flasks was inconvenient, this method was not used routinely. Methods of harvesting and purifying the polysaccharide will be described in detail below.

A synthetic medium was used in a limited survey of the kinds of carbon compounds utilized for growth and polysaccharide formation. The medium contained $(NH_4)_2SO_4$, 0.10%; $MgSO_4.7H_2O$, 0.02%; $FeCl_3.4H_2O$, 0.0005%; $CaCl_2$, 0.005%, plus 0.5–1.0% carbon compound. Growth in this medium was recognized by development of turbidity after incubation for up to one week at 30° C. Polysaccharide formation was recognized by examination of the centrifuged cell mass for the typical gelatinous character of growth. The cells were washed and tested with anthrone reagent for carbohydrate.

Methods of Analysis

Polysaccharide samples taken for analysis were dried *in vacuo* over $CaCl_2$ at 60° C. Air-dried samples usually contained 2–3% moisture.

Total carbohydrate in hydrolyzed and unhydrolyzed polysaccharide samples was determined by the anthrone method (14). The color values obtained were compared with a standard curve prepared with glucose.

Polysaccharides were hydrolyzed with 1 *N* H_2SO_4 at 100° C for 5 hours, and the reducing value of hydrolyzed samples was determined by the Nelson (16) colorimetric modification of the Somogyi method. Standard curves for glucose were prepared with each analysis and the results obtained from the samples were expressed as percentage of glucose.

Uronic acid was detected qualitatively by paper chromatography and quantitatively (on unhydrolyzed samples) by modification of the decarboxylation method of Maher (13) using the wet combustion apparatus described by Neish (15). The naphthoresorcinol method of Kapp (11) was used as an

occasional check of the amount of uronic acid in hydrolyzates. The carbazole method of Dische (9) was used as a qualitative test for uronic acids.

Hexosamines were estimated by the Blix modification of the Elson and Morgan procedure (2), on samples which had been hydrolyzed with 1 *N* HCl, at 100° C.

Total nitrogen was determined by the micro-Kjeldahl method. The ash content was estimated by incineration of polysaccharide samples in tared crucibles, to constant weight. Amino nitrogen was determined by the ninhydrin method of Rosen (18).

Glucosoxidase (Glucostat, Worthington Biochemical Co., Freehold, N. J.) and the cysteine – sulphuric acid reaction (8) were used as additional tests for identification and analysis of polysaccharide components.

Chemicals used for analysis were, in each instance, the best commercial grades. The carbohydrates were obtained from the Nutritional Biochemical Co., Chagrin Falls, Ohio, and from the California Corporation for Biochemical Research, Los Angeles, California. Organic reagents and solvents were obtained from the Distillation Products Division of the Eastman Kodak Co., Rochester, New York.

Turbidity readings and colorimetric analyses were done using a Bausch and Lomb Spectronic "20" colorimeter.

Chromatography of Acid Hydrolyzates

Hydrolyzates were applied to Whatman No. 1 filter paper and irrigated with the following solvent systems: ethyl acetate:pyridine:water (6:4:3 by volume), ethyl acetate:acetic acid:water (3:1:3 by volume), *n*-butanol: ethanol:water (5:1:4 by volume). Developed, dried chromatograms were sprayed with *p*-anisidine hydrochloride or aniline hydrogen phthalate for reducing sugars. Elson and Morgan spray reagents revealed the presence of amino sugars and ninhydrin revealed amino sugars and amino acids. These procedures and reagents were those suggested in the Manual by Bloch and his associates (3).

Recovery of the Gelatinous Polysaccharide

The extracellular polysaccharide was recovered with some difficulty from gelatinous cells. The cells were embedded in masses of tightly packed microfibers which appeared in flagella-stained smears examined microscopically (6). Various capsule staining methods when applied to the material met with little success, since no distinct capsule was resolved. Colonies of gelatinous strains growing on solid media could only be removed if forcibly peeled from the agar surface. Cell production on solid media was abandoned for this reason.

Cultures less than a week old, growing in broth, had all but traces of the polysaccharide bound to the cells. After incubation for from 4 to 6 weeks, however, much of the polysaccharide could be found free of the cells and was recoverable from the cell-free supernate. At this time, most of the cells were lysed and removal of debris from the polysaccharide was seemingly more

difficult than in the procedure described below. Inoculated liquid media in shaken flasks frequently turned to a non-fluid gum after 1 week and defied centrifugation. Stationary cultures, on the other hand, produced a tough surface membrane from which the underlying medium could be decanted, the membrane collected on a wire screen, and washed free of medium without much disruption. If an intact membrane failed to form, the gelatinous cells were harvested by centrifugation at 20,000 $\times g$ in a Lourdes model AX centrifuge, for 30 minutes in the cold. In either case the cell mass was subjected to alkaline extraction after pretreatment with 1% formaldehyde for 15 minutes, which according to Dudman and Wilkinson (10) reduced cell disruption. Figure 1 presents a scheme for the separation and purification of the gelatinous polysaccharide from the cells. The main problem was the removal of protein and other non-gelatinous polysaccharide materials. The first alcohol precipitation (4 volumes 95% ethanol) yielded the gelatinous polysaccharide, which could be collected as a ball on a glass stirring rod and removed. After 30 minutes in the cold, a flocculent material appeared which contained, in part, another polysaccharide. The latter was ordinarily discarded but occasionally was hydrolyzed and examined by paper chromatography as will

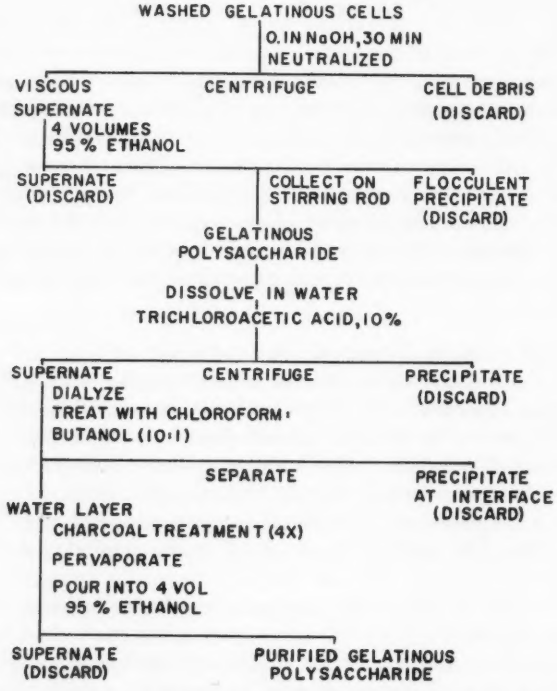

FIG. 1. Separation and purification of the gelatinous polysaccharide.

be described below. A flocculent polysaccharide but no gelatinous polysaccharide was detected from the cells of the non-gelatinous strains. No polysaccharide material was found in the medium in which these cells had grown after they had been removed by centrifugation.

Purification of the Gelatinous Polysaccharide

The gelatinous polysaccharide was freed of a considerable quantity of protein and brownish coloring matter, with trichloroacetic acid. The gelatinous polysaccharide was only slightly cloudy after dialysis to remove the acid. The preparation was subsequently made almost water clear by treatment with five separate portions of a chloroform (10 ml) and butanol (1 ml) or amyl alcohol (1 ml) mixture (19). Chromatography of hydrolyzates prepared from samples at this stage, however, still showed the presence of some amino nitrogen.

Much of the protein or peptide was further removed by passing the polysaccharide solution through a bed made of equal weights of activated charcoal and cellulose powder. Cifonelli and Mayeda (4) indicated that protein, peptides, nucleic acid, and nucleotides are strongly absorbed on charcoal which only very weakly absorbs large molecular weight polysaccharides. Passage of the solution through three more charcoal beds was necessary to completely remove the most persistent amino acid bearing residue, which contained two amino acids not yet identified. Following the treatment with charcoal the polysaccharide would not coagulate with alcohol (4 volumes 95% ethanol : 1 volume of polysaccharide solution) unless an electrolyte was added. A 1% water solution of sodium chloride was added dropwise with stirring to the alcoholic solution until coagulation occurred. The polysaccharide was separated and pressed into a thin layer on the wall of a beaker and dried under vacuum. The resulting polysaccharide was very tough, strong, and pliable. When the alcohol precipitate was pressed to remove most of the alcohol, then teased apart with tweezers, it appeared to be composed of many fine threads which when examined under the microscope were infinitely subdivided. The purified polysaccharide was quite readily soluble in water.

Results

The purified gelatinous polysaccharide accounted for 14–16% of the original cell dry weight. Table I compares the yield of polysaccharides from gelatinous cultures grown on three different media. A non-gelatinous culture was included as a control. The maximum yield of polysaccharide seemed to be dependent on the presence of amino nitrogen, and although carbohydrate was not absolutely essential for polysaccharide synthesis the yield was enhanced in the presence of carbohydrate. No increase in yield was obtained by increasing the glucose concentration. In general, any carbon compound which would support growth would support polysaccharide synthesis. Table II lists a number of carbon compounds that will and will not support growth and hence polysaccharide synthesis. This is not a comprehensive list but it does

TABLE I

The yield of gelatinous polysaccharide from *Chromobacterium* strains

Strain	Medium,* 100 ml	Volume of packed cells, ml	Total dry cell weight, mg	Polysaccharide yield		
				Crude, mg	Purified, mg	Per cent of cell dry weight
D_3G	P–G	34	147	74	27	18.4
	P	21	90	51	14	15.6
	S	10	42	26	6	14.3
30	P–G	38	150	72	24	16.0
32	P–G	36	155	78	25	16.1
D_3S†	P–G	Less than 1	160	0	0	0

*P–G, 1% peptone – 1% glucose broth; P, 1% peptone; S, 1% glucose – 0.05% ammonium sulphate synthetic medium. See text for details.
†A non-gelatinous strain.

TABLE II

The carbon source in relation to growth and extracellular polysaccharide synthesis in a synthetic medium

	Carbon source*		
	Carbohydrates	Organic acids	Amino acids
Growth and polysaccharide synthesis	Soluble starch Maltose Glucose Fructose Mannose	Succinate Malate Pyruvate Citrate	Alanine Asparagine Glutamate Aspartate Serine Threonine
No growth	Galactose Sorbose Arabinose Ribose Xylose Glucosamine Glucuronate Galacturonate	Oxalate Glycolate Lactate Salicylate Tartrate Acetate	Glycine Lysine Arginine Tyrosine Tryptophan Phenylalanine Histidine Valine Leucine Isoleucine Methionine

*Carbohydrates (1%), salts of organic acids (0.5%), amino acids (0.1%) used as sole carbon source in the synthetic medium described in the text. Non-gelatinous strain D_3S showed growth without extracellular polysaccharide synthesis.

indicate the variety of substances that could be utilized by these cells which included low molecular weight compounds. It should be emphasized that the gelatinous cultures are at times quite unstable. Consequently they were transferred several times before inoculation into the experimental media to make certain they were in the gelatinous phase. Some of the factors which influence the selection and development of non-gelatinous mutants are under study and will be reported later.

Analysis of the Gelatinous Polysaccharide

Samples of purified polysaccharide were hydrolyzed in glass-stoppered tubes in a boiling water bath and duplicates removed at intervals for determination of reducing sugar and hexosamine which had been shown to be

present by preliminary tests. The reducing sugars measured as glucose reached a value of 68% after 1 hour, as is shown in Fig. 2. The release of hexosamine paralleled the appearance of total reducing group after 1 hour but there was no significant increase in either reducing sugar or hexosamine after 2–3 hours.

Estimations of anthrone carbohydrate were routinely lower than estimations of reducing sugars, which indicated that all of the latter do not yield anthrone color or at least only a small fraction of the color produced by glucose. One of these was a hexosamine and the other uronic acid. Neither of these substances yielded color with anthrone in 10 times the concentration found in the polysaccharide. Chromatography of hydrolyzates on paper

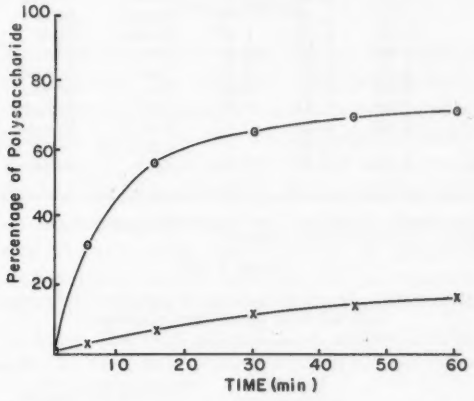

FIG. 2. Hydrolysis of gelatinous polysaccharide with 1 *N* HCl (100° C). Symbols: o, reducing sugar; ×, amino sugar.

strips using the acid solvent revealed the existence of four spots when the papers were sprayed with *p*-anisidine hydrochloride or aniline hydrogen phthalate spray reagents. These were, as is shown in Table III, a hexose sugar, uronic acid which gave two spots, and an amino sugar. The latter reacted with ninhydrin and Ehrlich's reagents.

Each of the components was isolated by elution from paper chromatograms and subjected to several qualitative tests, the results of which are shown in Table IV. On the basis of these color tests and chromatography the aldohexose is almost certainly glucose since none of the other common aldohexoses tested (galactose and mannose) reacted with glucosoxidase or gave the typical cysteine – sulphuric acid color reaction given by glucose and the unknown aldohexose. The color tests confirm the presence of a uronic acid and an amino sugar as component parts of the polysaccharide but their exact identity still remains in doubt.

An approximate analysis of the gelatinous polysaccharide is shown in Table V. The data indicate that about 90% of the total weight taken has

TABLE III

Paper chromatography of the gelatinous polysaccharide hydrolyzate and several reference sugars

Hydrolyzate sugar spots	$R_{glucose}$ values			Color development with:†			
	Solvent*						
	A	B	C	AH	AHP	N	EM
1	0.09	0.86	0.54	Red	Brown	—	—
2	0.73	0.62	0.65	Tan	Brown	Purple	Pink
3	1.02	1.04	1.00	Brown	Brown	—	—
4	—	1.99	—	—	Light	—	—
Reference sugars							
Glucose	1.00	1.00	1.00	Brown	Brown	—	—
Galactose	0.83	0.82	0.92	Brown	Brown	—	—
Mannose	1.15	1.14	1.30	Brown	Brown	—	—
Xylose	1.35	1.59	1.67	Brown	Pink	—	—
Rhamnose	1.60	2.00	2.00	Green	Tan	—	—
Glucosamine	0.72	0.60	0.68	Tan	Brown	Purple	Pink
Galactosamine	0.62	0.63	0.62	Tan	Brown	Purple	Pink
N-acetyl-glucosamine	1.40	1.44	1.50	Tan	Brown	Purple	—
Glucuronic acid	0.08	0.88	0.52	Red	Brown	—	—
	—	1.97	—	Light tan	Light yellow	—	—
Galacturonic acid	0.09	0.76	0.50	Red	Brown	—	—

*Solvents: A, ethyl acetate:pyridine:water (6:4:3); B, ethyl acetate:acetic acid:water (3:1:3); C, *n*-butanol: ethanol:water (5:1:4).
†Spray reagents: A, *p*-anisidine hydrochloride; AHP, aniline hydrogen phthalate; N, ninhydrin; EM, Elson–Morgan. A — sign indicates no colored spot was observed.

TABLE IV

Qualitative examination of the gelatinous polysaccharide components, isolated from paper chromatograms

Tests	Isolated component			Results*		
				Reference sugars		
	1	2	3	Glucuronic acid	Glucosamine	Glucose
Ninhydrin (18)	−	+	−	−	+	−
Elson–Morgan (2)	−	+	−	−	+	−
Naphthoresorcinol (11)	+	−	−	+	−	−
Carbazole (9)	+	−	−	+	−	−
Anthrone (14)	−	−	+	−	−	+
Glucosoxidase	−	−	+	−	−	+
Cysteine–H_2SO_4 (8)	−	−	+	−	−	+
Color after:						
5 min			Yellow			Yellow
48 hr			Green			Green

*The results are expressed as − or + values for convenience. The use of these tests in the identification of various sugars is described in detail by Ashwell (1) as well as by the specific reference cited above.

TABLE V

Partial analysis of the gelatinous polysaccharide from *Chromobacterium violaceum*

Analysis	Result*	Analysis	Result*
Carbohydrate	53.7–55.7%	Hexosamine	15.2–17.6%
Reducing value	68.5–70.0%	Amino nitrogen	1.6– 1.9%
Glucose	46.7–49.9%	Total nitrogen	1.9– 2.3%
Uronic acid	10.5–12.0%	Ash	1.2– 2.1%

*Determinations made after hydrolysis of the polysaccharide, except total nitrogen, uronic acid, and ashing which was done with oven-dried intact polysaccharide.

been accounted for. The anthrone value was usually higher than the specific analysis for glucose (glucosoxidase) which is somewhat difficult to explain since the other components present failed to give a test with either of these reagents.

Observations on the Flocculent Polysaccharide

Hydrolyzates of the flocculent polysaccharides were examined by paper chromatography. The $R_{glucose}$ values and the colors developed with the spray reagents previously described were compared with those of known sugars as is shown in Table III. This cursory examination suggested the presence of an aldohexose, a methyl pentose, and a still unidentified reducing sugar.

Discussion

Gelatinous *Chromobacterium violaceum* strains are common soil bacteria widely distributed in nature. Polysaccharide was produced by these organisms from simple carbon compounds plus ammonia as well as from several single amino acids serving as both a carbon and nitrogen source. Since the polysaccharide has pronounced resistance to microbial decomposition, its contribution to residual soil polysaccharides (24) is worth contemplating.

No information has been obtained to indicate the composition of the polysaccharide was affected either qualitatively or quantitatively when the cells were grown on different carbon compounds, but according to Wilkinson and his associates (23), the composition of Klebsiella polysaccharides was the same regardless of the sole energy and carbon source. Certainly the physical properties of the gelatinous polysaccharide were not obviously affected by the medium of growth.

A number of extracellular polysaccharides have been described in recent years (10, 18, 23), many of which have properties similar to those described here but most of these have a fairly distinct composition.

The characterization of the components of the gelatinous polysaccharide was confounded by the presence of large amounts of contaminating material, much of which was probably introduced as a result of the alkaline extraction method necessarily employed for removal of the gelatinous polysaccharide from the cells. One of the contaminants was a flocculent precipitate which was probably similar to the trichloroacetic acid soluble polysaccharide discovered by Davies (7) in strains of *Chromobacterium violaceum*. With the exception of one pair of strains, the polysaccharides showed little in common with one another. Similarity in the composition of the flocculent polysaccharide described in this paper to any of those described by Davies has not been established. A somewhat unique feature of the described TCA-soluble polysaccharides is the presence of certain heptose sugars, one of which has been identified as D-glycero-D-galacto-heptose (13).

Another contaminant of the gelatinous polysaccharide was the peptide residue bearing two still unidentified amino acids. This substance was not removed till after 3–4 passages of the polysaccharide through charcoal. This

suggested a much more intimate association than was true of most of the protein contaminants which were removed with less difficulty. It is doubtful that the peptide represented an enzyme residue, because in an enzyme residue one might expect to find a greater variety of amino acids.

The solubility of the gelatinous polysaccharide was greatly affected by its association with the cells. It adhered very tenaciously to them as evidenced by the fact that removal of young cells by centrifugation from the medium assured removal of the polysaccharide. After the polysaccharide had been extracted and purified of contaminating material, it became readily water soluble forming clear viscous solutions. Though the gelatinous polysaccharide may not be bound to the cell wall, it may be infused with antigenic polysaccharides associated with the cell wall (22) through a stronger type of bonding than afforded by weak physical attraction.

The formation of gelatinous polysaccharide seems to be an important biosynthetic feature of these cells but a property which can be lost and regained by some strains without affecting growth. Though no metabolic differences have yet been recognized between gelatinous and non-gelatinous strains, certainly the reactions leading to assimilation of carbon into the extracellular polymer or the ability to polymerize the sugar residues must be lacking in the non-gelatinous strains.

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EFFECT OF SOME METAL IONS ON THE GROWTH OF HALOBACTERIUM CUTIRUBRUM[1]

S. N. SEHGAL[2] AND N. E. GIBBONS

Abstract

Halobacterium cutirubrum, *H. salinarium*, and *H. halobium* required at least 10 p.p.m. ferrous ions for maximal growth in aerated cultures. The growth of *H. cutirubrum* was stimulated by 0.05 p.p.m. manganous ions but addition of divalent zinc or calcium had no effect on the growth of this organism.

Introduction

The red-pigmented extremely halophilic bacteria grow relatively slowly even in complex media containing fish infusion, gelatin, milk, or yeast extract. Weber (9) attempted to obtain a synthetic medium but found that gelatin hydrolyzate was necessary for heavy growth. Katznelson and Lochhead (3) found that the red halophiles did not require vitamins, purines, or pyrimidines but growth was stimulated by yeast extract and its ash. Brown and Gibbons (1) reported that most of the stimulation by yeast extract ash was the result of its potassium content and, using a casein hydrolyzate base, found that the red halophiles required 3.0–5.0 *M* sodium chloride, 0.1–0.5 *M* magnesium ion, 50–100 p.p.m. potassium ion, and 0.5–1.0 p.p.m. ferrous ion for maximal growth.

As a first step towards obtaining a synthetic medium for the growth of these organisms the effect of ferrous ion was reassessed and the effect of other bivalent metal ions on growth studied. This report describes the effects of iron on the growth of three red halophiles, *Halobacterium cutirubrum*, *H. salinarium*, and *H. halobium*, and of other metal ions on *H. cutirubrum*.

Materials and Methods

Media

The routine medium contained 7.5 g casamino acids (Difco), 10 g yeast extract (Difco), 3 g trisodium citrate, 2 g potassium chloride, 20 g magnesium sulphate heptahydrate, 250 g sodium chloride, and distilled water to 1 liter. The ingredients were dissolved in 800 ml water, the pH adjusted to 7.5–7.8 with *N* potassium hydroxide, and the medium autoclaved 5 minutes at 120° C. It was then filtered to remove the precipitate, the pH was adjusted to 7.4 with *N* hydrochloric acid, and the medium made up to final volume. Some 20% of the magnesium (see analysis below) and some phosphate are removed in the precipitate. However, sufficient of both ions remains in the medium for optimal growth. The basal medium, to study the effect of ions, was prepared in the same way but with reagent grade inorganic constituents

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Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada.
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[2]National Research Council Postdoctorate Fellow, 1958–60.

and demineralized distilled water. Analysis of this medium for ions gave the following results:[3] magnesium 0.16%, calcium 2 p.p.m., zinc 1 p.p.m., iron 0.5 p.p.m., manganese 0.03 p.p.m. Aliquots (50 ml) were dispensed into 250-ml Erlenmeyer flasks provided with side arms in which turbidity measurements could be made, and were sterilized at 120° C for 15 minutes. Flasks were plugged with non-absorbent cotton wrapped in cheesecloth. Iron was added as $FeSO_4.7H_2O$, calcium as $CaCl_2$, zinc as $ZnSO_4.7H_2O$, and manganese as $MnSO_4.H_2O$. All solutions were prepared in demineralized distilled water and were added without sterilization to the sterilized medium in Erlenmeyer flasks. The high salt concentration of the final medium prevented growth of organisms other than halophiles and microscopical examination indicated that there was no contamination.

Glassware for these tests was cleaned with a laboratory detergent followed by rinsing at least 10 times in each of tap water, distilled water, and demineralized distilled water. The flasks were then filled with demineralized distilled water and autoclaved for 30 minutes just before use (6).

Cultures

The test organisms were *H. cutirubrum* and *H. salinarium*, obtained originally from Dr. A. G. Lochhead, and *H. halobium*, from Dr. A. Pijper. Stock cultures were maintained on agar slants prepared by adding 2% agar to the routine medium.

Cells for inoculum were grown in the routine medium for 48 hours at 37° C, harvested by centrifugation, washed twice, and resuspended in 25% sodium chloride to give an optical density of 0.22 at 660 mμ. The standard inoculum was 0.5 ml of this suspension per flask. All test cultures were incubated at 37° C on a rotary shaker. Growth was measured as the turbidity at 660 mμ on a Coleman Junior spectrophotometer using uninoculated medium as the blank, and expressed as optical density.

Results

Effect of Iron on Growth

All three organisms grew poorly in the basal medium. When iron was chelated by adding 35 mg 2,2′-bipyridine per flask, there was no growth, thus confirming the earlier report of Brown and Gibbons (1). Attempts to chelate the iron quantitatively with bipyridine were not successful and therefore the effect of adding iron to the basal medium was studied. The addition of iron up to 50 p.p.m. increased the rate of growth of *H. cutirubrum* but amounts greater than 10 p.p.m. had little effect on the final population (Fig. 1). The effect of increasing amounts of iron on the growth of *H. cutirubrum* at 72 hours is shown in Fig. 2. *H. salinarium* and *H. halobium* reacted similarly to the addition of iron; the rate of growth was stimulated and the population increased (Fig. 2).

Since the optimal amount of iron indicated by these experiments is much greater than that found by Brown and Gibbons (1), an explanation was

[3]We are grateful to the Analytical Chemistry Section of the Division of Applied Chemistry for this analysis. The figures for iron and manganese are close approximations.

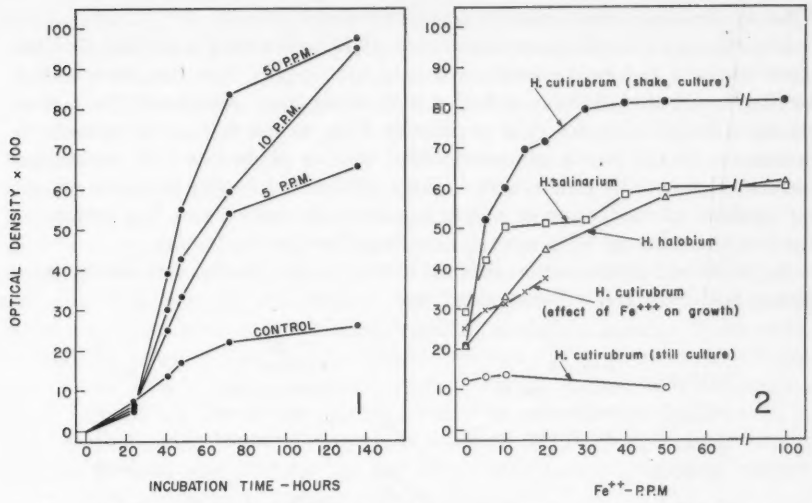

FIG. 1. Effect of concentration of ferrous iron on rate of growth of *Halobacterium cutirubrum*.

FIG. 2. Response of red halophilic bacteria to increasing concentrations of ferrous iron, of *H. cutirubrum* to ferric ion, and of *H. cutirubrum* in still and shaken cultures.

sought. In the earlier work the cultures were not shaken, so stationary cultures were compared with the shaken cultures. The results show (Fig. 2) that in stationary cultures the addition of 10 p.p.m. Fe^{++} increased growth slightly but by an amount comparable with that obtained by Brown and Gibbons; 50 p.p.m. Fe^{++} were actually slightly inhibitory. Under these conditions oxygen, evidently, is the limiting factor for growth.

Since it was doubtful that iron remained in the ferrous form under the conditions of growth, equimolar amount of ferric iron was added to the medium as $Fe_2(SO_4)_3$. In this medium a very slight stimulation of growth was obtained (Fig. 2) which could be accounted for by the ferrous impurities present in ferric sulphate.

The addition of chelating agents such as sodium citrate, versenol, and versenediol had no effect on iron utilization in citrate-free basal media. Whereas iron in the 2,2′-bipyridine–iron complex was unavailable for growth, iron chelated with citrate, versenol, or versenediol stimulated the growth of *H. cutirubrum* in citrate-free media to the same extent as ferrous ion alone.

In addition to stimulating the growth of *H. cutirubrum*, iron seemed to reduce the amount of sodium chloride required for growth. In the presence of iron growth was obtained in 14% salt medium whereas without added iron growth ceased below 15.4%. With 10 p.p.m. added iron the amount of growth obtained in 15.4% salt was comparable with that obtained in 25% salt without added iron.

A few preliminary experiments with Fe^{59} indicated a considerable amount of iron is taken up by the cells, most of which seems to be in the cytoplasm.

Effect of Manganese on Growth of H. cutirubrum

The effect of manganese on the growth of *H. cutirubrum* was studied in the basal medium and in the medium to which 10 p.p.m. iron had been added. In the former the addition of 0.05 p.p.m. manganese stimulated the rate of growth and increased the final population (Fig. 3). Increasing the amount of manganese to 0.5 p.p.m. almost doubled the lag phase but then stimulated growth. However, 2 p.p.m. was slightly inhibitory. In the presence of iron the optimal concentration of manganese was also 0.05 p.p.m. but increasing the concentration to 0.5 p.p.m. did not lengthen the lag period.

An increased pigmentation was noticeable in all cultures containing manganese both with and without added iron.

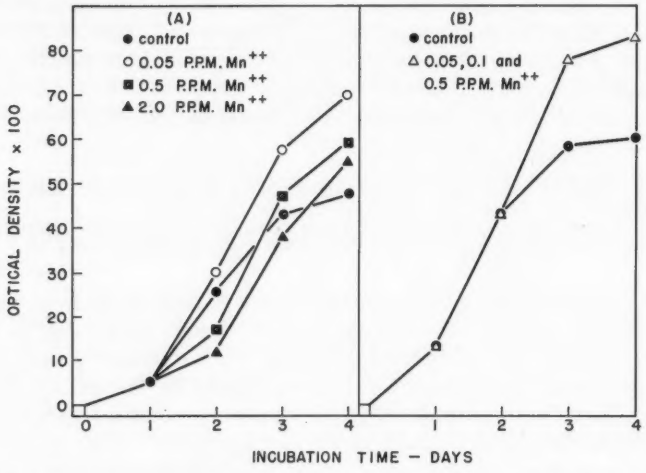

FIG. 3. Effect of concentration of manganese on rate of growth of *H. cutirubrum*. A—in basal medium. B—in basal medium + 10 p.p.m. Fe^{++}.

Effect of Zinc and Calcium on Growth of H. cutirubrum

Zinc added in concentrations from 0.05 to 2.0 p.p.m. had no effect on growth in the basal medium or in media containing 10 p.p.m. iron. Similarly calcium added in concentrations of 5 to 100 p.p.m. also had no effect on growth in either medium. The organic ingredients of the medium are reported to contain considerable amounts of calcium and zinc and analysis showed that 2 p.p.m. calcium and 1 p.p.m. zinc remain in the final medium. These amounts seem to be sufficient for optimal growth.

Discussion

Iron has been reported as an essential element for the growth of many bacteria. *Escherichia coli*, *Aerobacter aerogenes*, *Aerobacter indologenes*, and *Klebsiella pneumoniae* require an iron concentration of about 0.02 to 0.03 p.p.m. for maximal growth; *Pseudomonas aeroginosa* requires about four times

this amount (7, 8). *Serratia marcescens* has an even greater requirement, and production of the pigment prodigiosin is associated with the iron concentration (7, 8). MacLeod and Onofrey (4) reported that supplements of 0.005–0.01 p.p.m. of Fe^{++} in a sea water medium increased the rate and extent of growth of a marine bacterium investigated. The Fe^{++} requirement of the halophilic bacteria studied, therefore, is very high. Brown and Gibbons (1) reported a much lower requirement for the same organisms in stationary cultures. However, they pointed out that when cultures were shaken the amount of growth increased in presence of optimal amounts of magnesium; the same seems to be true of iron. In stationary cultures growth is limited by the diffusion of oxygen into the medium and therefore Fe^{++} utilization is limited. Since iron is an essential part of enzymes in oxidative systems of bacteria, it might be expected that more would be required and utilized when cultures were better aerated. Since sodium citrate, versenol, and versenediol do not affect iron utilization in our medium and it remains in ferrous form, it is possible that some constituents of our medium act as iron transport compounds. Demain and Hendlin (2) attributed such function to yeast extract and enzyme hydrolyzed casein in their recent paper.

The manganese requirement of *Halobacterium cutirubrum* is of the same order as that reported for many other organisms (5). The effect of Mn^{++} on production of pigment by this organism needs further investigation.

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THE VITAMIN REQUIREMENTS OF CORYNEBACTERIUM SEPEDONICUM (SPIEK. & KOTT.) SKAPT. & BURKH.[1]

ROBERT-A. LACHANCE

Abstract

The vitamin requirements of *Corynebacterium sepedonicum* (Spiek. & Kott.) Skapt. & Burkholder were studied in a medium containing vitamin-free acid casein hydrolyzate, glucose, and mineral salts. Nicotinic acid, thiamine, and biotin were found to be stimulatory. Riboflavin inhibited the growth slightly. Nicotinic acid can be replaced by nicotinamide, but Tween 80 and pimelic acid could not replace biotin.

Introduction

Conflicting reports have appeared in the literature on the vitamin requirements of *C. sepedonicum*, the causal organism of potato ring rot. Starr (3) reported that biotin, nicotinic acid, and thiamine were the "active" vitamins in the nutrition of *C. sepedonicum*. MacLachlan and Thatcher (1), however, found that calcium pantothenate caused the greatest increase in growth amongst nine vitamins tested. Thiamine, inositol, and para-aminobenzoic acid also caused a significant increase in the growth rate of the organism; however, when in combination with calcium pantothenate they failed to increase the rate of growth more than calcium pantothenate alone. The present investigation on the nutritional requirements of *C. sepedonicum* was undertaken in the hope of preparing a synthetic medium which would promote rapid growth of the organism and of shedding some light on the conflicting reports found in the literature concerning its vitamin requirements.

Methods

Cultures

Thirteen cultures of *C. sepedonicum* were used in these studies: four were obtained from Dr. H. Katznelson, Microbiology Research Institute, Ottawa; one from Dr. D. S. MacLachlan, Plant Research Institute, Ottawa; two from Dr. R. S. Dickey, Cornell University; one from Dr. P. A. Ark, University of California; one from Dr. W. J. Dowson, School of Botany, Cambridge, England; one from J. L. Howatt, Fredericton, N.B.; one from Dr. R. Bonde, Orono, Maine; one from Dr. E. Campagna, Ecole Supérieure d'Agriculture, Ste-Anne-de-la-Pocatière; and one isolated by the author.

Amongst the four cultures obtained from Dr. Katznelson one was A.T.C.C. No. 9850 and one was from Dr. M. P. Starr.

Inoculum

The stock cultures were maintained on potato dextrose agar at room temperature. Cells for inoculum were obtained by scraping a 72-hour slope

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Contribution from Canada Department of Agriculture, Research Laboratory, Ste-Anne-de-la-Pocatière, Québec.

culture. They were suspended in physiological saline and harvested by centrifugation, washed three times in saline, and resuspended in saline to give a light transmittance of about 90% at 600 mμ wave length in a "Spectronic 20" colorimeter (Bausch & Lomb). A volume of 0.1 ml was used to inoculate the various tests media.

Media

The medium used to establish the vitamin requirements had the following basic composition in grams per liter of distilled water: vitamin-free casein hydrolyzate (acid hydrolyzed) 5.0; KH_2PO_4 1.0; K_2HPO_4 1.0; $MgSO_4.7H_2O$ 0.2; $CaCl_2$ 0.1; $FeCl_3$ 0.02; $MnSO_4$ 0.03; NaCl 0.1; glucose 5.0. The vitamins were added to the basal medium either singly or in various combinations, to give the following concentrations in milligrams per liter of medium: thiamine HCl 1.0, riboflavin 1.0, inositol 1.0, biotin 0.002, choline 1.0, *p*-aminobenzoic acid 1.0, folic acid 0.02, calcium pantothenate 0.6, nicotinic acid 2.0, pyridoxine HCl 1.0. The casein hydrolyzate and the vitamins were obtained from the Nutritional Biochemicals Corporation.

The media were dispensed in matched test tubes 16×150 mm in 10.0-ml amounts and autoclaved for 10 minutes under 15 lb steam pressure.

The reaction of all media was adjusted to pH 6.8 before sterilization. Cultures were incubated at 23° C on a shaker rotating at approximately 160 r.p.m. Growth response was determined after 96 hours by measuring light transmittance at 600 mμ with the colorimeter. Uninoculated media served as control.

Results and Discussion

The study of the vitamin requirements of *Corynebacterium sepedonicum* made using the basal medium previously described demonstrated that nicotinic acid, thiamine, and biotin, although not essential in the strict sense of the word, are the critical vitamins in the nutrition of the organism (Table I). So far, growth has been sustained for 9 months in the basal medium free of vitamins. The 13 cultures studied required thiamine, nicotinic acid, and biotin for maximum growth. This observation is in agreement with that of Starr (3) but not MacLachlan and Thatcher (1), who reported that calcium pantothenate was the critical vitamin in the nutrition of *C. sepedonicum*. However,

TABLE I

Growth of *Corynebacterium sepedonicum* upon deletion of vitamins from medium

Vitamins deleted	% transmittance*	Vitamins deleted	% transmittance*
None	62.7	Inositol	65.3
Nicotinic acid	95.3	Choline	59.6
Pyridoxine HCl	60.3	PABA	64.0
Calcium pantothenate	63.3	Folic acid	64.2
Riboflavin	54.6	Biotin	68.2
Thiamine	92.6	All vitamins	96.6

*All values are the average of two determinations of five tubes each.

as an index of growth, the latter measured the oxygen consumption in the Warburg apparatus. According to Stokes (4) the results of MacLachlan and Thatcher indicate a rate of oxygen consumption "which is characteristic of non-proliferating cells rather than an increasing rate which one should expect in growing cultures". When the nutritional requirements of *C. sepedonicum* were tested by examining the effect of vitamin supplementation to the basal medium which already contained thiamine and nicotinic acid, results like those shown in Table II were obtained. The response to addition of biotin was more convincing than that obtained by the deletion experiment. The effect of biotin was to cause greater growth after 4 days.

TABLE II

Growth of *Corynebacterium sepedonicum* upon addition of vitamins to medium containing thiamine and nicotinic acid

Vitamins present	% transmittance*
None	98.0
Thiamine + nicotinic acid	54.2
Thiamine + nicotinic acid + biotin	34.2
Thiamine + nicotinic acid + PABA	55.0
Thiamine + nicotinic acid + inositol	54.0
Thiamine + nicotinic acid + riboflavin	58.7
Thiamine + nicotinic acid + pyridoxine acid	55.0
Thiamine + nicotinic acid + calcium pantothenate	54.5
Thiamine + nicotinic acid + choline	52.2
Thiamine + nicotinic acid + folic acid	52.8
All vitamins	48.0

*All values are the average of two determinations of five tubes each.

TABLE III

Effectiveness of Tween 80 and pimelic acid in replacing biotin

Vitamins added	% transmittance*
None	98.0
Thiamine + nicotinic acid	65.2
Thiamine + nicotinic acid + Tween 80†	75.6
Thiamine + nicotinic acid + pimelic acid†	71.2
Thiamine + nicotinic acid + biotin	54.0
Thiamine + nicotinic acid + biotin + Tween 80	71.0
Thiamine + nicotinic acid + biotin + pimelic acid	67.4

*All values are the average of two determinations of five tubes each.
†Concentration of Tween 80, 50 p.p.m.; pimelic acid, 20 p.p.m.

Tween 80 (polyoxyethylene sorbitate monooleate) (6) and pimelic acid (5) have been shown to replace biotin for certain microorganisms. The effect of adding these compounds to a medium devoid of biotin was determined. Tween 80 was tested at 100, 50, 20, and 10 p.p.m.; pimelic acid at 40, 20, and 10 p.p.m. Neither of these two compounds could replace biotin (Table III); they were not only ineffective in replacing biotin but they also suppressed the growth slightly.

Riboflavin, which is synthesized by *C. sepedonicum* (2), slightly inhibited the growth. Nicotinamide could replace nicotinic acid. The optimum concentration of thiamine, nicotinic acid, and biotin was found to be 0.6 mg, 2.0 mg, and 0.002 mg per liter respectively.

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THERMAL INACTIVATION OF MEF1 POLIOVIRUS[1]

R. C. FRENCH AND R. E. ARMSTRONG

Abstract

The thermal inactivation of MEF1 poliovirus in medium M150, or in phosphate-buffered saline, was found to proceed as a first-order reaction during the loss of more than 95% of the viral infectivity. The initial rate of virus inactivation was determined at several temperatures and the energies of activation, calculated from the Arrhenius equation, were found to be approximately 27,000 calories per mole over the temperature range from 4° C to 47° C, and about 200,000 calories per mole at temperatures above 47° C. The stabilizing effect of pretreatment of the virus with L-cystine was confirmed.

Introduction

When this investigation was begun practically no information was available on the stability of human strains of poliomyelitis viruses at various temperatures (5). Prior studies on the thermal inactivation of poliomyelitis viruses had been limited almost exclusively to the murine strains with energies of activation from 34,500 to 100,000 calories per mole being reported (1, 8). During an investigation of the tissue culture safety testing of Salk vaccines (3) it was necessary to study the stability of MEF1 poliovirus at 37° C in medium M150 (11) and for practical as well as theoretical reasons it was decided to study the rate of inactivation at other temperatures.

More recently Youngner (16) and Lycke (10) presented some data on the thermal inactivation of human strains of poliomyelitis viruses in medium 199 (12) and Pohjanpelto (13) reported on their stability in phosphate-buffered saline (PBS) after pretreatment with L-cystine. However, these studies have been confined to one or two temperatures or at best to a narrow temperature range. During the present study a different technique was utilized in order to measure initial reaction velocities. Over a wide range of temperatures in M150 and in PBS it was found that the initial loss of virus followed the course of a first-order reaction and that a single value for energy of activation would not suffice to describe the experimental data. Similar findings had been reported by Price (15) for the inactivation of tobacco mosaic virus and by different workers for some other viruses (10). Further investigation of the nature of thermal inactivation will undoubtedly be stimulated by the recent observation (9) that the attenuated strains of polioviruses used in oral vaccines show a greatly reduced ability to propagate at higher temperatures as compared with the virulent strains.

Materials and Methods

The stock preparation of MEF1 poliovirus used in this investigation was prepared by adding 2×10^8 plaque forming units (pfu) of virus to a week-old monolayer culture of monkey kidney cells in a Roux bottle containing 100 ml

[1]Manuscript received November 16, 1959.
Contribution from the Virus Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

of fresh M150. After incubation for 20 hours at 37° C cellular debris was removed from the fluid by sedimentation for 30 minutes at 2000 r.p.m. in an International Centrifuge, size 2. The clarified virus suspension, containing $10^{7.8}$ pfu per milliliter, was then dispensed in sterile ampoules which were sealed and stored at −50° C.

The general procedure adopted in determining the thermal inactivation of virus was as follows. One milliliter of a 1/100 dilution of the stock virus in M150 was added to 99 ml of medium which had been prewarmed in a stoppered Roux bottle to the selected temperature in a water bath constant to within ±0.5° C. The initial virus concentration in the various experiments averaged 3100 pfu per 0.5 ml, the observed values of 1500 to 4000 pfu resulting chiefly from variation in the sensitivity of the assay cultures. After thorough mixing, 1-ml amounts were removed by pipette at selected time intervals and quickly discharged into 4-ml amounts of M150 at 4° C. At the lower temperatures studied the initial virus concentration was determined on "zero-time" samples removed within 2 minutes after the addition of virus, but at higher temperatures initial virus was estimated on samples obtained from controls in which 1 ml of a 1/100 dilution of the stock virus had been added to 99 ml of M150 at room temperature. When it was desired to follow the destruction of virus beyond 99%, undiluted samples were immediately chilled by immersion in a beaker containing ice water, and titrated for pfu without further dilution. After the last sample of a series was taken, all of the 1/5 dilutions were removed from storage at 4° C and 0.5-ml amounts of appropriate further dilutions in M150 were assayed for pfu on monolayer cultures of monkey kidney cells in T-60 flasks. The method of assay was previously described in more detail (2).

Since the estimation of the amount of virus initially present may occasionally ($P=0.05$) be in error by as much as ±25% (2), it was found necessary in most experiments to plot the logarithm of the surviving pfu against time and to draw a regression line best fitting all the data. The initial virus concentration was then calculated from the intercept on the ordinate at "zero-time" and all other observed values were expressed as percentages of this initial virus concentration. Being chiefly concerned in these investigations with the fate of the major portion of the virus, the average values for the first-order reaction velocity constants at various temperatures presented in this work were based on the inactivation of at least 97% of the initial virus, and the slower rates applying to the inactivation of the remaining 3%, or less, were not regularly followed.

In most of the inactivation studies in M150 no attempt was made to achieve the difficult control of the pH of the medium within narrow limits. Amounts of 100 ml of M150 in stoppered Roux bottles usually started at a pH of around 7.3 and rose to a pH of about 8 in the longer experiments, owing to a loss of CO_2, averaging approximately pH 7.7. In several experiments at 37° C the medium was adjusted daily with *N* HCl to pH's as low as 6.7. In other experiments the rate of inactivation was studied at pH 7.2 in PBS, or in M150 adjusted to pH 9.0 with *N* NaOH.

Results

Inactivation of MEF1 Virus in Medium M150

The data for a typical inactivation experiment at 32° C are presented in Table I. The graphical representation of these data and of those for a similar experiment at 37° C is presented in Fig. 1. Within experimental error, both curves indicate that the course of destruction of more than 98% of the virus follows that of a first-order reaction with velocity constants of 2.2×10^{-4} and 5.0×10^{-4} min^{-1} for 32° C and 37° C, respectively. In more prolonged experiments at 37° C it was found that the logarithmic rate of inactivation persisted until more than 99.5% of the virus had been inactivated, after which the remaining virus was destroyed at a slower logarithmic rate.

TABLE I

Inactivation of MEF1 poliovirus at 32° C*

Days at 32° C	(1) Dilution of virus, 1:	(2) Average plaques per 0.5 ml	(3) Plaques per 0.5 ml undiluted	Log_{10} per cent plaques surviving
0	80	21.67	1734	2.00
2	40	20.50	820	1.67
4	20	20.75	415	1.38
6	10	25.25	253	1.16
8	5	33.50	168	0.99
10	5	12.50	62.5	0.56
13.7	Undiluted	26.75	26.8	0.19

*In M150 at approximately pH 7.6.
(1) Diluted after exposure at 32° C.
(2) Average for 4 to 6 cell cultures.
(3) Average plaques × dilution factor.

The data obtained for three separate experiments at 52° C are presented in Fig. 2. This figure shows the maximum variation found between the several experiments at this temperature. Such variations tended to be greater at the higher temperatures investigated. The inactivation of approximately 97% of the virus followed a first-order course, after which the remaining virus was inactivated at a slower logarithmic rate.

The results of the inactivation studies in M150 are presented in Table II, and represent many of the data shown in Fig. 3. Each reaction velocity constant presented is the average value obtained in from two to eight separate experiments. The value listed for 4° C was based on the following data. Two suspensions of MEF1 virus in M150 were each assayed five times using different cultures of monkey kidney cells. The average values of each five-test assay were 4.61×10^5 and 267 pfu per 0.5 ml, respectively. After storage for 6 months at 4° C in stoppered test tubes, each virus suspension was again assayed five times and average values of 2.34×10^5 and 99 pfu per 0.5 ml were obtained for the respective solutions. If it be assumed that the loss of virus occurred as a first-order reaction at 4° C, then the values of the reaction velocity constants could be calculated as 2.57×10^{-6} and 3.81×10^{-6} min^{-1}. The average value of 3.2×10^{-6} min^{-1} appears to be in good agreement with

the value predicted from the inactivation rates observed over the temperature range from 27° to 42° C (Fig. 3, curve A). At 57° C the rate of inactivation was too rapid to obtain a reliable constant by the technique used in these studies.

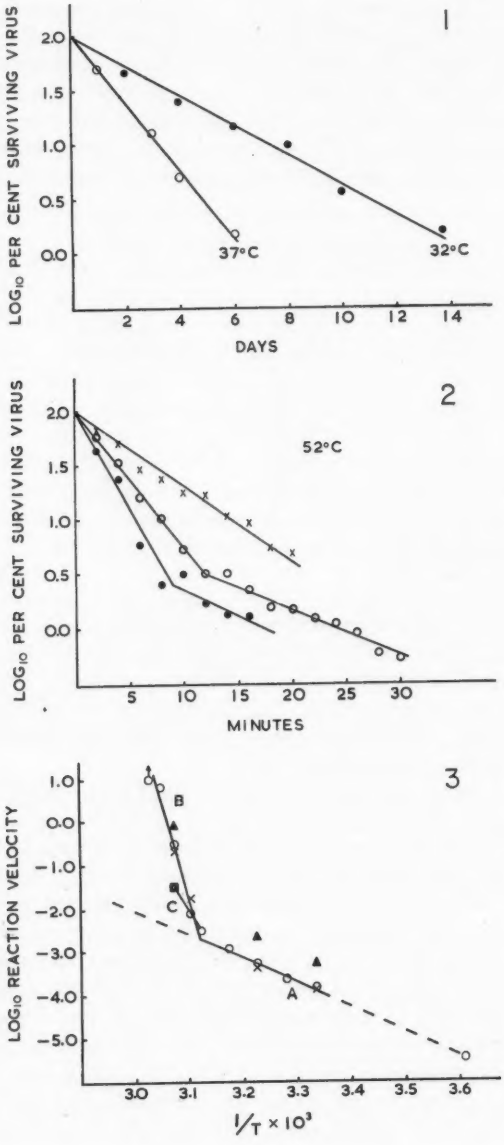

TABLE II

Inactivation rates for MEF1 poliovirus*

Degrees Centigrade	$\frac{1000}{T}$	Half-life in minutes	K†	$\text{Log}_{10} K$
4	3.610	2.17×10^{5}	3.2×10^{-6}	−5.50
27	3.333	4.95×10^{3}	1.4×10^{-4}	−3.85
32	3.279	3.01×10^{3}	2.3×10^{-4}	−3.64
37	3.226	1.65×10^{3}	5.0×10^{-4}	−3.30
42	3.175	6.30×10^{2}	1.1×10^{-3}	−2.95
47	3.125	2.39×10^{2}	2.9×10^{-3}	−2.54
49	3.106	8.67×10	8.0×10^{-3}	−2.10
52	3.077	2.65	2.9×10^{-1}	−0.54
55	3.049	1.05×10^{-1}	6.6	0.82
57	3.030	$\lesssim 6.90\times10^{-2}$	>10	>1.0

*In medium M150 at pH 7.6.
†K=reaction velocity constant per minute. Each K value represents the average of two to eight separate tests.

When the logarithms of the average reaction velocity constants were plotted against the reciprocals of the absolute temperatures, the data for the inactivation of MEF1 virus in M150 (open circles in Fig. 3) were best fitted by lines A and B rather than the usual single straight line. The energy of activation calculated from the Arrhenius equation gave a value of 26,600 calories per mole over the temperature range from 4° C to 47° C (curve A, Fig. 3), while a value of 202,000 calories per mole was obtained over the range from 47° C to 55° C (curve B, Fig. 3).

Inactivation of MEF1 Virus at Various pH Values

It was at first considered that the change of slope at or around 47° C might be related to a change in the pH of the medium. Accordingly inactivation rates for the virus in PBS at pH 7.2 were studied using the technique previously employed. The results obtained at four selected temperatures are also presented in Fig. 3. These results, each the average of two or more experiments, are in good agreement with those found for M150 and hence the high energy of activation observed at temperatures above 47° C is not related to changes in pH.

In several experiments at 37° C the pH of the medium M150 was adjusted daily with N HCl down to pH's as low as 6.7. No consistent or significant change in the reaction velocity constant for inactivation was observed. When the medium was adjusted to pH 9.0 with N NaOH, the values represented

FIG. 1. Thermal inactivation of MEF1 poliovirus in medium M150 at pH 7.6. Solid circles represent values obtained in an experiment at 32° C, open circles represent values obtained at 37° C.

FIG. 2. Thermal inactivation of MEF1 poliovirus in M150 at pH 7.7. The values obtained in three separate experiments at 52° C are represented in the figure.

FIG. 3. Thermal inactivation of MEF1 poliovirus. The logarithm of the reaction velocity constant is plotted on the ordinate against the reciprocal of the absolute temperature on the abscissa. Curves A and B represent the values obtained in M150 (○) at pH 7.6 or in PBS (×) at pH 7.2. The values obtained in M150 at pH 9, or for cystine-stabilized virus, are represented by (▲) and (■), respectively.

in Fig. 3 by triangles were obtained. Although these constants are considerably higher than those obtained at lower pH's, they still require a two-line rather than a single-line fitting of the data.

The Stabilizing Effect of L-Cystine

Pohjanpelto (13) reported that a heat-sensitive strain of poliovirus was greatly stabilized against thermal inactivation by pretreatment with L-cystine or L-cysteine. Although M150 contains L-cystine (20 μg per milliliter) an additional 80 μg per milliliter was added and the mixture incubated for 16 hours at 37° C in order to achieve maximum stabilization. The technique used was identical with that described by Pohjanpelto except that M150 was used instead of PBS. After pretreatment with L-cystine thermal inactivation tests were performed at 52° C. In several experiments the stabilizing effect of L-cystine was invariably observed. This effect could not be attributed to the prior destruction of the more labile virus particles during the 16-hour incubation at 37° C, since a loss of only 20% of the virus was observed as a result of this treatment. Pretreatment of the virus with lower concentrations of L-cystine, or for shorter time intervals, resulted in virus of intermediate stability. The most stable preparations obtained gave a reaction velocity constant for inactivation of 3×10^{-2} min^{-1} at 52° C, represented by the solid square in Fig. 3, as compared with the average of 2.9×10^{-1} min^{-1} obtained for untreated virus. This value still lies considerably above curve A, extrapolated from the lower temperature points, and represents an activation energy of at least 91,600 calories per mole (curve C, Fig. 3).

Discussion

The energies of activation of 26,600 calories per mole at 27–47° C and 92,000 to 202,000 calories per mole at 47–55° C found in the present study are compatible with the conclusion of Youngner (16) that different mechanisms of inactivation were operative at 36.5° C and 50° C, heat-resistant variants being regularly segregated at the higher but not at the lower temperature. Lycke (10) also reported that the energy of activation at 50° C was considerably higher than the 17,000 calories per mole which he found in the temperature range from 7 to 37° C.

It was suggested by Lycke (10) that the relatively low energy of activation which he observed over the range from 7 to 37° C may be dependent upon oxidation as the rate-determining factor and this suggestion does not conflict with Pohjanpelto's (13) finding that the thermal stability of poliovirus was much greater in the absence of oxygen. However, the somewhat higher value of 27,000 calories per mole which we have obtained in the present work may not be incompatible with Pollard's (14) suggestion that the slow process of viral infectivity loss is due to the inactivation of nucleic acid. Little is known about the thermal denaturation of nucleic acids, but recently a value of 37,000 calories per mole was obtained for the denaturation of calf thymus DNA (6).

The relatively high energy of activation found in the present work at temperatures above 47° C is usually associated with the denaturation of proteins (4, 7), and may reflect the more extensive breaking of the cross-linkages such as disulphide or hydrogen bonds believed to hold proteins in specific configuration. It has been shown by du Vigneau that the biological activity of insulin is dependent upon the disulphide linkage of cystine (4) and Pohjanpelto (13) suggested that the specific effect of cystine "stabilizes the (polio) virus by combining with the SH groups of the virus protein and by subsequently producing cross-linkages in the protein".

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STUDIES WITH STAPHYLOCOCCAL TOXINS

V. POSSIBLE IDENTIFICATION OF ALPHA HEMOLYSIN WITH A PROTEOLYTIC ENZYME[1]

J. ROBINSON, F. S. THATCHER, AND JEANNINE MONTFORD

Abstract

Alpha hemolysin has been separated from many of the other components present in culture filtrates of four strains of *Staphylococcus aureus* using a column chromatographic procedure with carboxymethyl-cellulose as the selective adsorbent, and graded levels of phosphate buffer at pH 6.0 to provide selective elution. The preparation of alpha hemolysin obtained in this manner hemolyzed rabbit and sheep erythrocytes, induced a dermonecrotic reaction in rabbits, was lethal to mice, and was proteolytic. The hemolytic activity of the preparation was stimulated by ethylenediamine tetraacetic acid, presumably by removing toxic ions from solution. Divalent cations inhibited activity of the alpha hemolysin, but stimulated the activity of a distinctive sheep hemolysin, which is shown to be a separate entity.

Alpha hemolysin, obtained by the chromatographic procedure, and subjected further to resolution by zone electrophoresis retained its capacity to induce dermonecrosis, to hemolyze rabbit erythrocytes, and to hydrolyze casein. Apparent loss of the capacity of the preparation to hemolyze sheep erythrocytes was discussed.

The possibility that alpha hemolysin is a specific proteolytic enzyme is suggested by the observation that under no conditions could a separation be effected between the hemolytic and proteolytic properties of the alpha hemolysin recovered from a number of different cultures.

Introduction

The capacity of *Staphylococcus aureus* to produce a substance which hemolyzes rabbit erythrocytes has been related to the pathogenicity of these organisms (9, 25); other investigators (12, 22) have found no such relationship. The dermonecrotic and lethal properties of culture filtrates of *S. aureus* have been associated with their alpha hemolysin activity (5, 6, 16, 23). Parker (27) and Weld and Gunther (31) found that the capacity of a culture filtrate to hemolyze rabbit erythrocytes was reduced to negligible proportions by treatment with erythrocyte stroma but that this treatment was without effect on the activity of the dermonecrotic toxin. On the basis of these observations it was concluded that dermonecrosis and hemolysis were effected by different toxic entities. Lack (21), without published experimental evidence, supported this thesis. The capacity of some culture filtrates of *S. aureus* to kill test animals has been linked with alpha hemolysin (5, 16). Data obtained by Burky (4) and Butler (7) suggested that this relationship may be invalid. Fulton (15) and Brown, Prichard, and Quilliam (3) suggested that a relationship exists between alpha toxin and enterotoxic activities of culture filtrates, but this seems to be rather tenuous when the data obtained by Dolman and Wilson (10), Matheson and Thatcher (26), Bergdoll (2), and others are considered. One of the properties of alpha hemolysin produced in culture filtrate

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by *S. aureus* is to hemolyze rabbit and sheep erythrocytes at 37° C (8). Flaum and Forssman (13), however, claim to have found two monovalent hemolysins, one of which hemolyzes specifically the erythrocytes of sheep while the other is effective specifically against rabbit erythrocytes.

Clearly, much confusion exists in the literature about the biological properties of the so-called alpha hemolysin. The present work was initiated, therefore, with the thought that useful information about this hemolysin could be obtained following its separation from the unrelated components normally present in culture filtrates of *S. aureus*. In this paper, the methods will be described which were used to prepare electrophoretically homogeneous alpha hemolysin. Investigations to examine the possibility that the alpha hemolysin is a specific proteolytic enzyme will also be reported.

Materials and Methods

The strains of *Staphylococcus aureus* used in the present study were coagulase positive. Most of the data presented in the current report have been obtained using the same strain L-16 used in previous experiments (30). Strains designated as numbers 588415 and 588426 of clinical origin, and of phage pattern 80/81, were obtained from Dr. E. T. Bynoe, Laboratory of Hygiene, Ottawa.

Culture filtrates containing a variety of exotoxins were prepared by inoculating the semisolid medium described by Dolman and Wilson (10) using broth cultures of the appropriate strains which were 5 hours old. The cultures, incubated 3 days at 37° C in an atmosphere containing initially 10% CO_2 and 90% O_2, were harvested and the residuum of cells of *S. aureus* removed by passage through a Seitz filter pad.

Treatment of the culture filtrate with ethyl alcohol at −20° C, calcium phosphate gel, and ammonium sulphate according to the methods used previously in this laboratory (30) resulted in the isolation of preparations which hemolyzed sheep but not rabbit erythrocytes. The ability of the culture filtrate to hemolyze rabbit erythrocytes was maintained, however, by dialyzing it against cold running tap water for 18 hours and by lyophilizing the resulting solution. The material prepared in this manner was used as a source of crude hemolysin.

Initially, the fraction which hemolyzed rabbit erythrocytes was separated from some of the other constituents of the culture filtrate using calcium phosphate gel. Subsequently, however, a more complete resolution of the constituents of the culture filtrate could be effected using a column chromatographic procedure with carboxymethyl-cellulose (CMC) prepared according to the method detailed by Peterson and Sober (28). The chromatographic column was prepared in a 25-ml burette and contained CMC suspended and washed several times with 0.0156 *M* phosphate buffer at pH 6.0. A 100-mg sample of lyophilized culture filtrate was dissolved in 2.0 ml of the same buffer and the resulting solution clarified by centrifuging it 30 minutes in a refrigerated unit at 12,000 r.p.m. A 1.0-ml aliquot of the supernatant solution

was then applied slowly to the surface of the CMC column. Elution of the various components of the sample was accomplished by a simple stepwise elution procedure using three concentrations of phosphate buffer adjusted to pH 6.0. Samples (3.5-ml aliquots) were collected using an automatic fraction collector manufactured by Gilson Medical Electronics, Madison, Wisconsin, U.S.A.

Some of the fractions separated by the chromatographic procedure were lyophilized and subjected to further resolution by the zone electrophoresis procedure described by Kunkel and Slater (20) for which granules of potato starch were used as the inert supporting medium and tris buffer or acetate buffer at various pH levels in the continuous phase. Intact granules of potato starch were separated from a commercial product obtained from Hatfield Industries Limited, Hartland, N. B., using the procedure described previously (30). The starch block was prepared in a trough constructed from polyvinyl plastic 15 mm wide, 8 mm deep, and 80 cm long. Electrophoretic separation, under an applied potential of 350 v, was continued for 18 hours at room temperature, after which time the starch block was cut into 50 sections 1.5 cm in width and each extracted with 3.0 ml of tris buffer (0.025 *M*) at pH 7.4.

Dermonecrosis was evaluated by injecting intradermally into normal rabbits 0.2-ml aliquots of the test material. Preliminary results had indicated that the diameter of the necrotic area developing in 5 to 7 days, expressed in millimeters, was related to the quantity of toxic material injected. For purposes of this paper, dermonecrotic activity has been expressed on this basis. Lethality was determined by the occurrence of death of mice within 5 hours after intraperitoneal injection.

Proteolytic activity was determined by the method of Anson (1) as modified by Husain and McDonald (18). The substrate was prepared by suspending 9.0 g of vitamin-free casein in 150 ml of water, and to this adding 150 ml of 0.05 *N* NaOH. The mixture was boiled for 10 minutes, cooled, and to it was added 50 ml of a solution containing 3.0 g of tris buffer (tris(hydroxymethyl) aminomethane) and 0.1 g merthiolate (Lilly). The pH was adjusted to 7.4 with dilute hydrochloric acid and the volume adjusted to 450 ml.

The reaction mixture for the assay of enzyme activity contained 3.5 ml of substrate, 1.0 ml of the solution to be tested, and 0.1 ml of toluene. Protein was precipitated from solution initially and after 18 hours of incubation at 37° C by mixing equal volumes of 9.8% trichloroacetic acid and test solution (1.5 ml each). The level of tyrosine present in solution was determined using the method of Folin and Ciocalteau (14). Proteolytic activity was expressed as the amount of tyrosine liberated under the conditions of the test by 1 μg of enzyme protein.

Hemolysin activity of all test preparations was determined by a colorimetric procedure which is based on the estimation of the quantity of hemoglobin liberated into solution following hemolysis in buffers containing tris (0.0128 *M*), NaCl (0.153 *M*), and KCl (0.0048 *M*). $MgSO_4.7H_2O$ was added as required to a final concentration of 6.0×10^{-4} *M*. The soluble hemoglobin, expressed as hemin, was determined by the method of Rimington (29). Blood

for this purpose was collected aseptically and mixed directly with a solution containing 2.3 g of glucose and 1.7 g of sodium citrate in 100 ml of solution at the rate of three volumes of blood to one volume of sterile solution. Sheep and horse blood was drawn from the jugular vein, rabbit blood by heart puncture of anaesthetized animals, and human blood by vena puncture. The erythrocytes of each were washed three times and suspended in a solution containing 0.9% NaCl and 0.036% KCl at the rate of 4.0 ml of packed cells to 100 ml of solution.

Protein was determined by the colorimetric method developed by Lowry, Rosebrough, Farr, and Randall (24).

Results

The results illustrated in Fig. 1 are typical of the many obtained when an aliquot of the culture filtrate of *S. aureus* strain L-16 was applied at the surface of a column containing CMC, and the components separated by the chromatographic procedure, using 0.0156 *M* phosphate buffer at pH 6.0 as the initial eluant. The bulk of the protein and pigment was flushed through the column with this level of buffer. The protein of this fraction was unable to hemolyze the erythrocytes of sheep, rabbit, horse, or man under any of the conditions tested, but was strongly dermonecrotic. This non-hemolytic, dermonecrotic material has several interesting properties which will be discussed in a subsequent paper. After 90 ml of 0.0156 *M* phosphate buffer

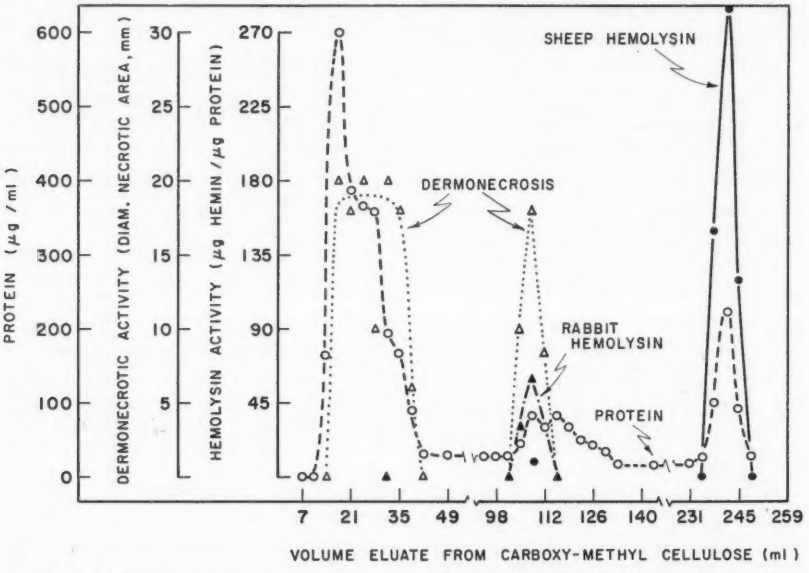


FIG. 1. Separation by means of the column chromatographic procedure of the protein constituents in 50 mg of lyophilized culture filtrate from strain L-16 using successively 0 0156 *M*, 0.045 *M*, and 0.25 *M* phosphate buffer at pH 6.0.

had been eluted through the chromatographic column, the concentration of phosphate in the buffer was increased to the 0.045 *M* level, a second fraction was collected which was dermonecrotic and was 10 times as active against rabbit erythrocytes as it was against sheep erythrocytes. Results obtained subsequently showed that it was also lethal and possessed proteolytic activity. This fraction has all the characteristics which Glenny and Stevens (16) and others have attributed to alpha hemolysin. For this reason, it will be referred to here by this name even though proteolysis is not a property previously associated with this toxin. When the concentration of phosphate buffer was increased again, this time to the 0.25 *M* level, a third fraction was collected which hemolyzed sheep erythrocytes only and evoked no dermonecrotic reaction. This fraction has the properties of the "sheep hemolysin" described by Robinson, Thatcher, and Gagnon (30).

Several tests were conducted to determine whether any difference could be detected in the manner in which sheep erythrocytes were hemolyzed by alpha hemolysin and the specific sheep hemolysin. The possibility was entertained that the fraction isolated by the chromatographic procedure which hemolyzed rabbit erythrocytes and to a more limited extent those of sheep, and tentatively designated as alpha hemolysin, was contaminated with a small portion of the fraction which had the capacity to hemolyze sheep erythrocytes exclusively. Results presented in Table I show that lysis of rabbit and sheep erythrocytes by alpha hemolysin was reduced to a limited extent by the metallic ions tested. The lysis of sheep erythrocytes by the specific sheep hemolytic fraction isolated by the chromatographic procedure was activated, on the contrary, by the ions of magnesium, manganese, and

TABLE I

Comparative effect of metallic ions on the activity of alpha hemolysin and sheep hemolysin isolated by the chromatographic procedure

Ionic additions (50×10^{-5} *M*)	Hemolysin activity (μg hemin/μg protein)	
	Sheep erythrocytes	Rabbit erythrocytes
Alpha hemolysin		
Co^{++}	10.6	78.7
Ca^{++}	10.0	130.3
Fe^{++}	Nil	Nil
Zn^{++}	Nil	Nil
Mg^{++}	11.4	118.2
Mn^{++}	12.5	112.1
None	11.4	136.3
Sheep hemolysin		
Co^{++}	118.4	Nil
Ca^{++}	Nil	Nil
Fe^{++}	Nil	Nil
Zn^{++}	Nil	Nil
Mg^{++}	36.9	Nil
Mn^{++}	63.2	Nil
None	8.0	Nil

TABLE II

Comparative effect of magnesium (2.5×10^{-4} *M*) and ethylenediamine tetraacetic acid (6.25×10^{-4} *M*) on the activity of alpha hemolysin and the specific sheep hemolysin

	Hemolysin activity (μg hemin/μg protein)			
	Rabbit erythrocytes		Sheep erythrocytes	
	Mg added	No Mg	Mg added	No Mg
Alpha hemolysin				
With EDTA	30.0	26.0	3.0	3.4
Without EDTA	22.0	18.0	0.2	0.8
Specific sheep hemolysin				
With EDTA	Nil	Nil	10.0	Nil
Without EDTA	Nil	Nil	8.5	6.0

cobalt. Results presented in Table II show that EDTA stimulated the activity of alpha hemolysin, but inhibited the activity of the specific sheep hemolysin. Inclusion of magnesium in the reaction mixture reversed the inhibition of the latter hemolysin by EDTA. The capacity of alpha hemolysin to effect lysis of sheep erythrocytes, therefore, would appear to have no relationship to its contamination with traces of sheep hemolysin.

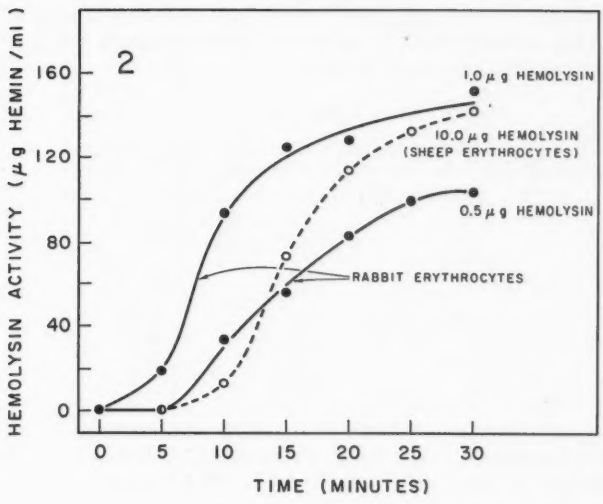
Further information was obtained to support this observation. The extent to which activity of alpha hemolysin was related to time and to hemolysin concentration was also determined. The results show that a relationship existed between hemolysin activity and time (Fig. 2) and between hemolysin activity and hemolysin concentration (Fig. 3) which was characterized by a typical sigmoid curve rather than by a straight-line relationship as was the case with the sheep hemolysin (30).

Data are presented in Table III which show to what extent alpha hemolysin activity was affected by pepsin and trypsin. The probability that alpha hemolysin is a protein is suggested by the observation that its activity was destroyed by both proteolytic enzymes but was unaffected by dialysis.

TABLE III

Effect of pepsin and trypsin on the rabbit hemolytic activity of alpha hemolysin obtained by the chromatographic procedure (hemolysin and proteolytic enzymes incubated 2 hours at 37° C)

	Hemolytic activity (μg hemin/μg protein)
In tris buffer, pH 8.0	
Toxin with trypsin	Nil
Toxin with buffer only	6.1
Trypsin with buffer only	Nil
Buffer only	Nil
In acetate buffer, pH 4.6	
Toxin with pepsin	Nil
Toxin with buffer only	4.7
Pepsin with buffer only	Nil
Buffer only	Nil



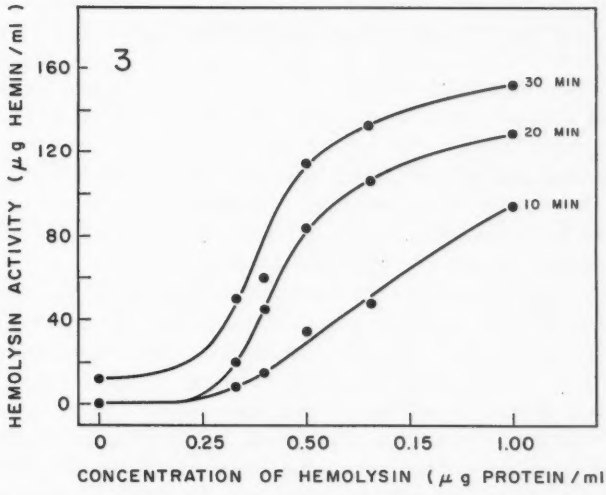


FIG. 2. Hemolysis of rabbit and sheep erythrocytes by graded levels of alpha hemolysin from strain L-16 separated from other components of the culture filtrate by the chromatographic procedure. Toxin dissolved and erythrocytes suspended in tris buffer at pH 7.4 and containing 0.9% NaCl and 0.036% KCl.

FIG. 3. Hemolysis of rabbit erythrocytes by graded levels of the same preparation of alpha hemolysin used for Fig. 2, under similar experimental conditions.

Results presented in Table IV show that alpha hemolysin separated by the chromatographic procedure from many of the contaminating materials present in culture filtrates was more susceptible to destruction at 60° C than at 100° C. This anomalous observation has been reported by Kodama and Nisiyama (19) and others, cf. Elek (11).

TABLE IV

Effect of heat on the rabbit hemolytic activity of alpha hemolysin isolated by the chromatography procedure

Time of exposure (minutes)	Hemolytic activity (μg hemin/μg protein)	
	60° C	100° C
Initial	4.9	4.9
5	2.0	4.1
10	0.71	5.6
15	0.57	3.0
30	0.15	—

By the application of the zone electrophoresis procedure, attempts were made to resolve preparations with typical alpha hemolysin activity into constituents which possessed properties more restricted than those noted in the original material isolated chromatographically. Preparations obtained from culture filtrates of strain L-16, and which hemolyzed rabbit erythrocytes, retained their proteolytic activity and their capacity to induce dermonecrosis in rabbits when they were subjected to zone electrophoresis using acetate buffer at pH 5.0 and tris buffer at pH 7.4. Results presented in Fig. 4 show that with acetate buffer at pH 4.3 a second minor component of the alpha hemolytic fraction was detected which was dermonecrotic but had no hemolytic properties, although the major hemolytic component still retained its capacity to induce dermonecrosis and to hydrolyze casein. None of the fractions obtained by this procedure were able to hemolyze sheep erythrocytes.

Several strains of *S. aureus* were examined to determine if the relationship found to exist between alpha hemolysin and a proteolytic enzyme in strain L-16 applied also to alpha hemolysin produced by other strains of this species. The capacity of various preparations of alpha hemolysin to hemolyze the erythrocytes of sheep and rabbits, to hydrolyze casein, and to induce a dermonecrotic reaction in rabbits was determined. The results, presented in Table V, show that the activity of the hemolysins subjected successively to chromatographic separation and zone electrophoresis was invariably lower than that of preparations subjected to the one manipulation of chromatography. The results also show that the fractions isolated by the chromatographic procedure were able to hemolyze sheep erythrocytes but that the same preparations subjected subsequently to zone electrophoresis lost this capacity. All specimens of alpha hemolysin which were examined and which were obtained directly as a result of chromatography or by the successive application of chromatography

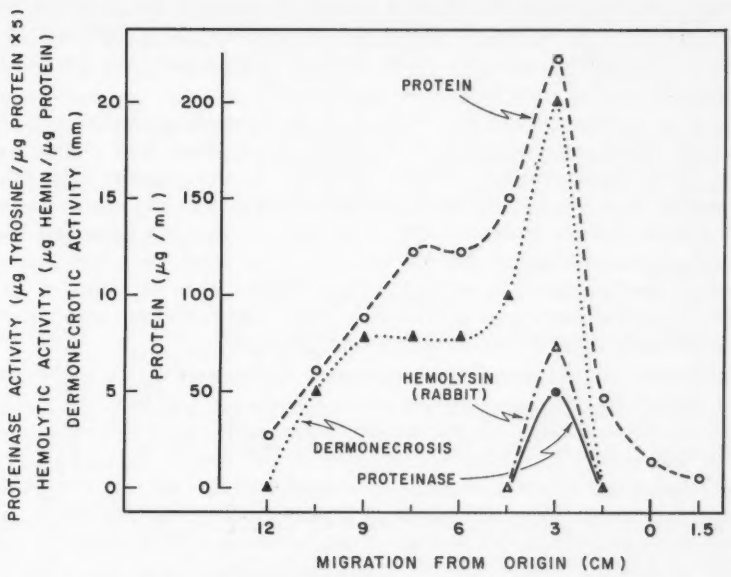


FIG. 4. Separation, by the zone electrophoresis procedure, of the protein constituents of a preparation of alpha hemolysin obtained from culture filtrates of strain L-16 by column chromatography. Electrophoretic separation accomplished in 0.025 *M* acetate buffer at pH 4.3.

TABLE V

Activity of preparations of alpha hemolysin produced by several strains of *S. aureus* and separated from other components in the culture filtrate by the chromatographic and zone electrophoresis procedures

Strain of *S. aureus* employed	Preparative procedure*	Hemolytic activity†		Proteolytic activity‡	Dermonecrotic activity, mm
		R	S		
L-16	C	11.2	0.8	0.84	—
L-16	C	88.0	10.0	0.8	40
L-16	C	84.0	1.9	—	35
L-16	C-E				
	pH 4.3	7.0	0	0.96	20
	pH 4.0	2.8	0	0.68	25
	pH 7.35	18.2	0	—	12
588415	C	2.6	0.23	0.6	30
588426	C	7.0	0	0.89	25

*C, preparations obtained by chromatographic procedure.
C-E, preparations obtained by the successive application of chromatography and zone electrophoresis.
†Hemolytic activity with rabbit (R) and sheep (S) erythrocytes expressed as μg hemin/μg protein.
‡Proteolytic activity expressed as μg tyrosine liberated into solution from casein/μg enzyme.

and zone electrophoresis contained a proteolytic enzyme. Disparity between the comparative activities of hemolysis and proteolysis is believed to be related to the differences in the lapse of time between carrying out the two determinations. This was technically unavoidable.

Much of the confusion in the literature concerning the properties exhibited by specific toxic entities produced by *S. aureus* can be traced to the use of crude culture filtrates from a variety of strains of this organism and failure to consider that similar reactions may be effected by more than one component of the culture filtrate. In the present paper, data are presented which show that a non-hemolytic fraction as well as the alpha hemolysin are able to induce dermonecrosis in rabbits. It is suggested that attempts to relate specific biological properties exhibited by crude culture filtrates with a particular constituent can lead to erroneous conclusions.

Specific reactions induced by components in culture filtrates also may be either inhibited or activated by unknown constituents, and this, too, can lead to still further confusion. In the present paper, as in paper IV of this series, EDTA was shown to inhibit the activity of the specific sheep hemolysin, while stimulating activity of the alpha hemolysin. Specific metallic cations, on the other hand, activated the sheep hemolysin, but inhibited the activity of the alpha hemolysin. Activation of the latter by EDTA has been interpreted as indicating that specific toxic cations have been removed from solution.

Activity of proteolytic enzymes in preparations obtained from culture filtrates of *S. aureus* may be evaluated quantitatively by determining the amount of tyrosine liberated into solution following hydrolysis of casein. This method has been applied in the present work to a determination of the proteolytic activity of preparations with alpha hemolysin activity. The results presented in this paper show that under none of the experimental conditions examined using four different cultures could the alpha hemolysin and proteolytic activity of a preparation be separated. This relationship between the capacity of staphylococcal alpha hemolysin to hemolyze rabbit erythrocytes and a specific proteolytic enzyme, we believe to be a new finding.

Rabbit erythrocytes have been shown to be unaffected by pepsin at pH 4.0 or by trypsin at pH 8.0. It is known, too (18), that micrococci may produce proteolytic enzymes without producing hemolysin. Activated coagulase without hemolytic activity has been shown (17) to possess some properties of a proteolytic enzyme. Data are not yet available to show to what extent these non-hemolytic proteolytic enzymes differ from the one which apparently is related to alpha hemolysin.

Alpha hemolysin, separated from many of the other components of culture filtrates of *S. aureus* by the chromatographic procedure, was lethal, dermonecrotic, hemolytic to rabbit and sheep erythrocytes, and was proteolytic. When preparations with these properties were subjected subsequently to zone electrophoresis a minor non-hemolytic but dermonecrotic component was separated from the major component. All biological properties of the major

component were reduced in degree by the electrophoretic treatment. The properties of hemolysis of rabbit erythrocytes, dermonecrosis, and proteolysis were retained, but the capacity to hemolyze sheep erythrocytes and to kill mice was reduced to a level that rendered detection of these properties uncertain. These properties were not present in any other fraction. In the absence of convincing experimental evidence to the contrary,. the present indication seems to be that lethality and the hemolysis of sheep erythrocytes are associated with the component which also hemolyzes rabbit erythrocytes, is dermonecrotic, and is proteolytic.

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STUDIES WITH STAPHYLOCOCCAL TOXINS

VI. SOME PROPERTIES OF A NON-HEMOLYTIC DERMONECROTIC TOXIN PRODUCED BY SOME STRAINS OF STAPHYLOCOCCUS AUREUS[1]

J. Robinson, F. S. Thatcher, and Jeannine Montford

Abstract

A non-hemolytic, dermonecrotic preparation with proteolytic activity was isolated from culture filtrates of some strains of *Staphylococcus aureus* using a chromatographic procedure with carboxymethyl-cellulose as a selective adsorbent. The preparation was resolved into components which were independently proteolytic and dermonecrotic using a zone electrophoresis procedure.

The non-hemolytic dermonecrotic toxin possessed properties which characterize it as a protein. No relationship could be found between pathogenicity of the staphylococcus and its capacity to produce the toxin.

The presence in some culture filtrates of the non-hemolytic, dermonecrotic toxin has been considered as a possible explanation for the lack of agreement noted by several authors between alpha hemolytic and dermonecrotic titers.

Introduction

The capacity of culture filtrates of *Staphylococcus aureus* to induce a dermonecrotic reaction in rabbits or guinea pigs has been associated with the alpha hemolysin produced by this organism (2, 5). This has recently been confirmed for human skin by Lawrence (10). Another hemolytic agent produced by the staphylococcus, the α_2-hemolysin (13) or δ-hemolysin (12), is also able to induce a dermonecrotic reaction. The capacity of a culture filtrate of *S. aureus* to hemolyze rabbit erythrocytes was reduced to negligible proportions using erythrocyte stroma as a selective adsorptive agent (18), but this treatment had little or no effect on the capacity of the preparation to induce dermonecrosis. On the basis of this evidence, Weld and Gunther (18) concluded that alpha hemolysin was not dermonecrotic. Lack (9), without published experimental evidence, tentatively concurred with this conclusion. Data presented by Avery, Rigdon, and Johlin (1) showed that the dermonecrotic activity of a culture filtrate could be inhibited by lithium chloride; hemolytic activity of the same preparation was unaffected by exposure to this salt. None of the foregoing investigators seem to have considered the possibility that a multiplicity of toxic components with dermonecrotic activity may be produced by *S. aureus*; in none of the reports is the possibility entertained that different toxic entities may be affected differently by various procedures.

Robinson, Thatcher, and Montford (17) have shown that several distinct protein fractions are produced in culture filtrate by *S. aureus*, strain L-16. Two of these were shown to be dermonecrotic, although only one was hemolytic. The hemolytic protein resembled the classical alpha hemolysin described by Glenny and Stevens (5), but in addition was also proteolytic. As far as we

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Contribution from the Food Microbiology Section, Food and Drug Directorate, Department of National Health and Welfare, Tunney's Pasture, Ottawa, Ontario.

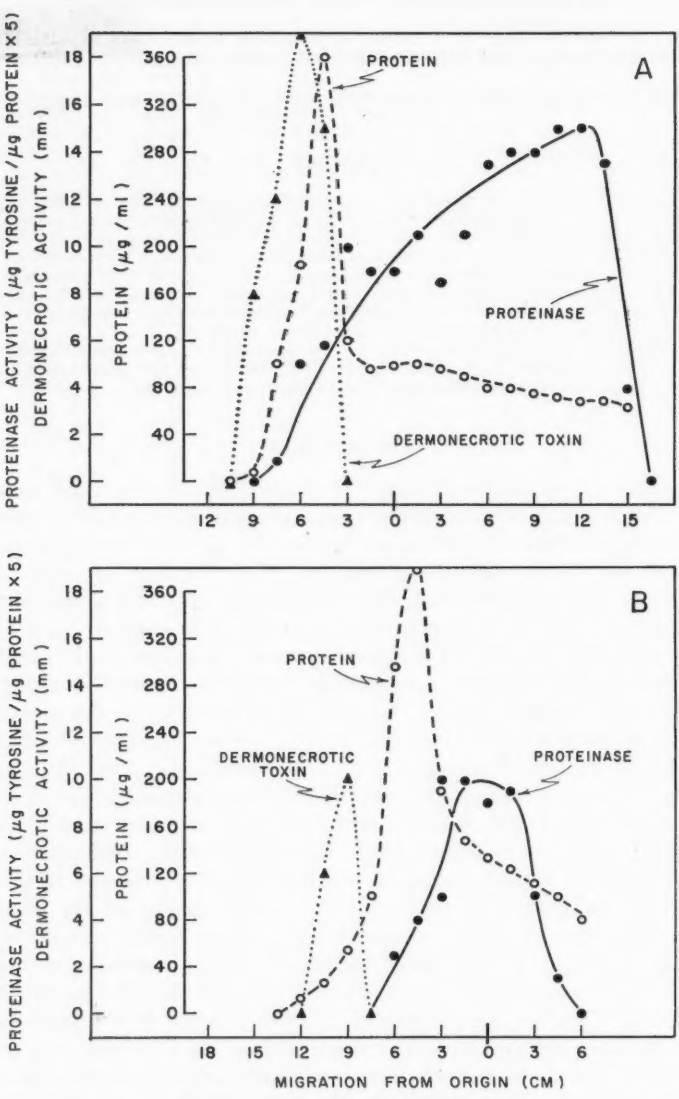

FIG. 1. Separation, by the zone electrophoresis procedure, of the protein constituents of a preparation containing the non-hemolytic dermonecrotic toxin in association with a proteolytic enzyme. The preparation was obtained from a culture filtrate of strain L-16 by column chromatography. Electrophoretic separation accomplished (A) in 0.025 *M* tris buffer, pH 7.2, (B) in 0.025 *M* acetate buffer, pH 5.0.

have been able to tell, a non-hemolytic, dermonecrotic toxin has not previously been described. In the present paper, some properties of the latter toxin and studies to determine any relationship between this substance and pathogenicity will be described.

Materials and Methods

The following strains of *S. aureus* were used in the present study: L-16, isolated in this laboratory from cheese incriminated in an outbreak of food poisoning, had the following phage pattern, 42C/47B/47C/81+; cultures labelled as 588415, 588426, 588383, and 588455 were clinical isolates of phage type 80/81 obtained from Dr. E. T. Bynoe, Laboratory of Hygiene, Ottawa; the strain known as Wood 46 was obtained from the Department of Bacteriology, McGill University.

Culture filtrates of the different strains of *S. aureus* were obtained by inoculating each onto the surface of a semisolid medium (4) and incubating 72 hours at 37° C in an atmosphere containing 10% CO_2 and 90% O_2 before separating the cells from the filtrate by centrifugation. All culture filtrates were dialyzed 18 hours against cold running tap water, sterilized by Seitz filtration, and lyophilized.

Lyophilized culture filtrates were reconstituted as required in 0.0156 *M* phosphate buffer, pH 6.0, and fractionated with the aid of a column chromatographic procedure, using carboxymethyl-cellulose according to the methods outlined by Peterson and Sober (16), and also by a zone electrophoresis procedure using granules of potato starch by the method employed by Kunkel and Slater (8). The hemolytic, dermonecrotic, and proteolytic activities of the fractions isolated by these procedures were assessed by methods described earlier (17). The level of protein present in solution was determined by a colorimetric procedure (11).

The non-hemolytic, dermonecrotic toxin was prepared from culture filtrates of *S. aureus*, strain L-16, using the chromatographic procedure outlined by Robinson *et al.* (17). This fraction was lyophilized and used as required.

Results

A preliminary examination of the fraction with non-hemolytic, dermonecrotic properties showed it to be proteolytic when examined by the procedure employed by Husain and McDonald (7). Attempts were made, using the zone electrophoresis procedure, to resolve it into components which were independently proteolytic and dermonecrotic. The data presented in Fig. 1A show that with tris buffer (tris(hydroxymethyl)aminomethane) adjusted to pH 7.2 some separation could be effected between the dermonecrotic and proteolytic components of the original preparation; at pH 5.0 (Fig. 1B) complete separation was effected.

Data obtained by Parfentjev, Clapp, and Waldschmidt (15) showed that the hemolytic and dermonecrotic properties of alpha hemolysin produced by the Wood 46 strain were completely destroyed by treatment with pepsin and

trypsin in appropriate buffers during incubation for 24 hours at 37° C. To determine to what extent the non-hemolytic, dermonecrotic toxin was affected by these two proteolytic enzymes, aliquots of the toxic preparation were added to 0.025 *M* acetate buffer at pH 5.0 with and without pepsin and to 0.025 *M* tris buffer at pH 8.0 with and without trypsin. These mixtures, with appropriate controls containing buffer alone, and pepsin or trypsin in their respective buffers, were incubated 2 hours at 37° C and the mixtures then injected intradermally into the back of a rabbit. A photograph of the back of an animal receiving the injections is reproduced as Fig. 2. These results show that neither of the proteolytic enzymes, pepsin and trypsin, nor the two buffers were dermonecrotic. Both proteolytic enzymes, under the conditions of the test, almost completely destroyed the dermonecrotic properties of the preparation and this, together with the fact that activity of the preparation was unaffected by dialysis, suggests that the non-hemolytic, dermonecrotic toxin is a protein.

Much importance has been attached by previous workers to the response to heat (either 60° or 100° C) of the alpha and beta hemolysins. Aliquots of a non-hemolytic, dermonecrotic preparation with proteolytic activity were heated for 20 minutes at 60° C and 100° C. The results are presented in Table I and show that all dermonecrotic activity was destroyed when the preparation, suspended in physiological saline, was heated to 60° C. It is interesting to note that not all the activity was destroyed by bringing the preparation to 100° C. Results similar to these with the dermonecrotic fraction have been obtained in our own laboratory (17) as well as in other laboratories with alpha hemolysin.

TABLE I

Effect of heat on the dermonecrotic activity of the non-hemolytic, dermonecrotic toxin isolated from culture filtrates of strain L-16 by the column chromatographic procedure

Sample	Dermonecrotic area, diameter in mm
Untreated	20
Heated 20 min at 60° C	0
Heated 20 min at 100° C	10

The dermonecrotic activity of the same preparations was determined after incubation at refrigeration temperatures for 6 days. Results presented in Table II show that an apparent increase rather than a decrease in activity

TABLE II

Effect of storage at 5° C on the dermonecrotic activity of the same preparation employed for Table I

Incubation period, days	Dermonecrotic area, diameter in mm
Initial	20
1	30
6	30

FIG. 2. Effect of pepsin and trypsin on the dermonecrotic activity of the non-hemolytic, dermonecrotic toxin isolated from a culture filtrate of strain L-16 by the chromatographic procedure.

In tris buffer, pH 8.0: 1, toxin with trypsin; 2, toxin with buffer only; 3, trypsin with buffer only; 4, buffer only.

In acetate buffer, pH 4.6: 5, toxin with pepsin; 6, toxin with buffer only; 7, pepsin with buffer only; 8, buffer only.

In tris buffer, pH 7.2: 9, 10, toxin with buffer only.

was noted during this period. This may pose a question as to whether part of the toxic molecule may be present in an inactive form or as a protoxin which could be activated by a proteolytic enzyme.

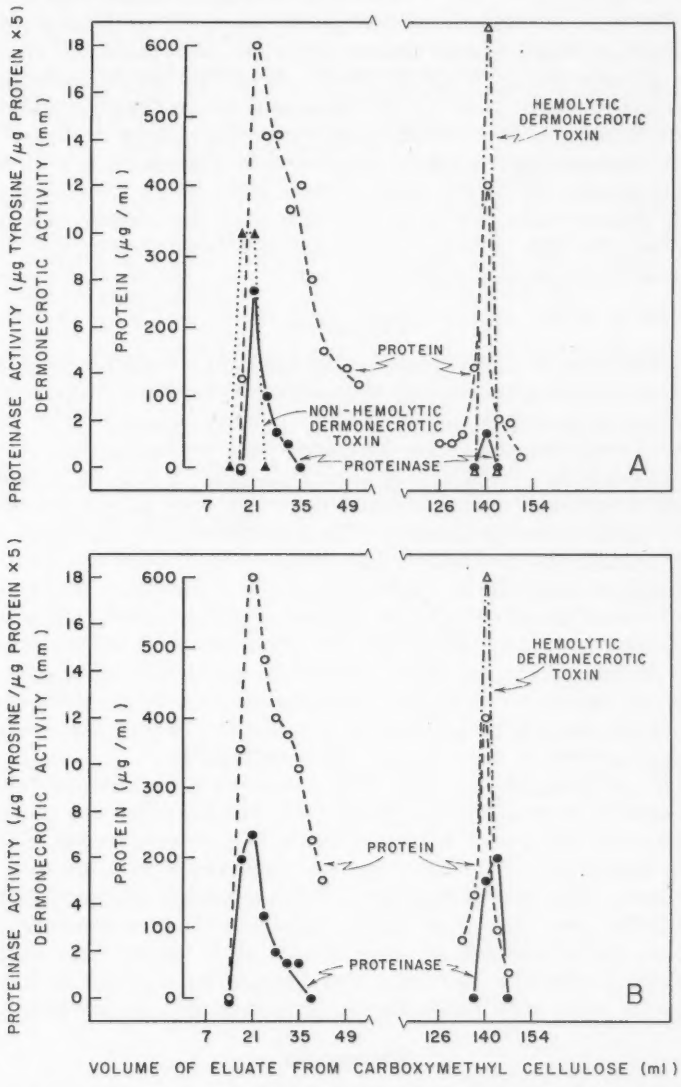

FIG. 3. Separation by means of the column chromatographic procedure of the protein constituents in 50 mg of lyophilized culture filtrate using successively 0.0156 *M*, 0.045 *M*, and 0.25 *M* phosphate buffer at pH 6.0. (A) Filtrate from culture number 588415, (B) filtrate from culture number 588426.

Much has yet to be learned about the factors which are conducive to the pathogenicity of staphylococci. It seemed desirable to learn whether the non-hemolytic dermonecrotic toxin was consistently produced by virulent strains. To determine if such a relationship existed, attempts were made to isolate the toxin by column chromatography from culture filtrates of four strains of type 80/81 recently isolated from clinical sources and also from culture filtrates of the Wood 46 strain. A non-hemolytic dermonecrotic fraction was separated from culture filtrates of strains 588415, 588383, and L-16 but a fraction with similar properties could not be separated from filtrates of cultures 588426, 588455, and Wood 46. Figures 3A and 3B express data from strains 588415 and 588426, respectively, which are typical of the different productivities in relation to this specific dermonecrotoxin. A proteolytic enzyme was isolated from comparable eluates from the culture filtrates of all strains of *S. aureus* examined.

Discussion

The production of a non-hemolytic, dermonecrotic toxin by some strains of *S. aureus* could explain the lack of parallelism noted by some investigators (3, 14) between hemolytic and dermonecrotic titers in specific crude culture filtrates. It might also explain the observation made by Weld and Gunther (18) that a culture filtrate containing alpha hemolysin lost its capacity to hemolyze rabbit erythrocytes when it was treated with erythrocyte stroma, but retained much of its dermonecrotic property. The data presented in this paper would seem to point to the probability that erroneous conclusions can be reached when biological properties of a culture filtrate of *S. aureus* are examined and assumed to be those of one specific component of the filtrate. Only after the various components of a culture filtrate have been separated and the properties of each determined can particular reactions be associated with specific components of the filtrate. The fact that the non-hemolytic, dermonecrotic toxin was not consistently produced by virulent strains of *S. aureus* suggests that the toxin does not play a major role in pathogenesis.

Heckly and Nigg (6), working with a dermonecrotic toxin produced by *Pseudomonas pseudomalleii*, and Zellar (19), who examined the proteolytic properties of snake venom, suggested that a positive relationship may exist between the proteolytic and dermonecrotic properties of the toxin from these two sources. Preliminary data obtained in this laboratory suggested that such a relationship may also have been applicable to the non-hemolytic, dermonecrotic toxin produced by some strains of *S. aureus*. This suggested relationship was found to be invalid after a separation of the preparation into constituents which were independently proteolytic and dermonecrotic.

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DEVELOPMENT OF FINE STRUCTURE, THERMOSTABILITY, AND DIPICOLINATE DURING SPOROGENESIS IN A BACILLUS[1]

TADAYO HASHIMOTO,[2] SAMUEL H. BLACK,[3] AND PHILIPP GERHARDT

Abstract

A correlative study was made of some structural and functional features of a reasonably synchronized culture of *Bacillus cereus* strain terminalis during spore formation and maturation. The successive stages of development could be recognized by phase contrast microscopy and in electron micrographs of ultrathin-sectioned cells. Attempts were made to correlate these changes with the acquisition of heat resistance and the synthesis of dipicolinic acid. The outer coat of the spores was observed to be formed first around the forespore; the exosporium, cortex, and inner coat then appeared sequentially and independently of existing sporangial membranes. Dipicolinic acid synthesis began in the early transitional stage, just after forespore formation, and reached one third of the maximum level before an increase of heat resistance in the population was detectable, indicating the possibility of a correlation only above a threshold level of the compound.

Introduction

The extraordinary properties of bacterial spores increasingly appear to be related not only to peculiar biochemical components and processes but also to a distinctive anatomy. An integrative study of these two features seemed likely to provide new information that otherwise might be missed. This approach was employed by us in a subsequent study (1) to analyze the basis for the heat susceptibility and deficiency of dipicolinic acid (DPA) of spores formed "endotrophically" in water, and it also has been directed to problems of spore permeability (2), DPA localization (8), and morphogenesis in other sporulating organisms (9, 11).

In the experiments reported below, efforts were focused on some details of sporogenesis in a commonly used aerobic sporeformer, *Bacillus cereus* strain terminalis.[4] A new technique of preparing ultrathin sections for electron microscopy (10) together with other accepted methods of observation and analysis were brought to bear on the following interrelated problems: (1) the origin, formation, and characteristics of spore membranes; (2) spore refractility and stainability; (3) the synthesis of DPA; and (4) the acquisition of heat resistance.

Materials and Methods

Mass Culture

A phage-resistant mutant was isolated from a culture of *B. cereus* strain terminalis in the stock culture collection at The University of Michigan.

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[4]There is some question concerning the correct terminology and species status for this organism, variously referred to as *Bacillus terminalis*, *Bacillus cereus* var. *terminalis*, *Bacillus cereus* strain T, etc. Because of its widespread use in studies on aerobic spores, we have preferred to retain some identification with the descriptive name, "terminalis", using it as a strain designation in deference to legitimate classification (17).

Rapidly dividing cells were subcultured twice on trypticase-soy agar and then seeded into 1 liter of modified G-medium[5] contained in a 2-liter Erlenmeyer flask. This culture, incubated on a rotary shaker at 30° C for 8 hours so that only filamentous cells were present, was used to inoculate 14 liters of modified G-medium contained in a 16-liter Pyrex carboy. Aeration and agitation were accomplished by admitting sterile air at the rate of 40 liters/minute through two submerged inlets of 2-mm inside diameter. Dow-Corning antifoam AF was added to control excessive foaming. The effluent air was passed through two condenser flasks and a heated pyrex-wool filter before being released to the atmosphere. Samples were removed periodically by means of a sampling line fed by the positive pressure in the carboy. In this system, sporulation of the population progressed in a relatively uniform fashion and became completed by the 24th hour after carboy inoculation.

Microscopy

A dark phase contrast microscope, equipped with 35-mm photomicrographic accessories, was used for routine examination. Ultrathin sections of cells were prepared for electron microscopy by the agar-enclosing technique devised by Hashimoto and Naylor (10). A Porter-Blum ultramicrotome with a glass knife was used for sectioning; examination was made with an RCA EML-1A electron microscope.

Assay Procedures

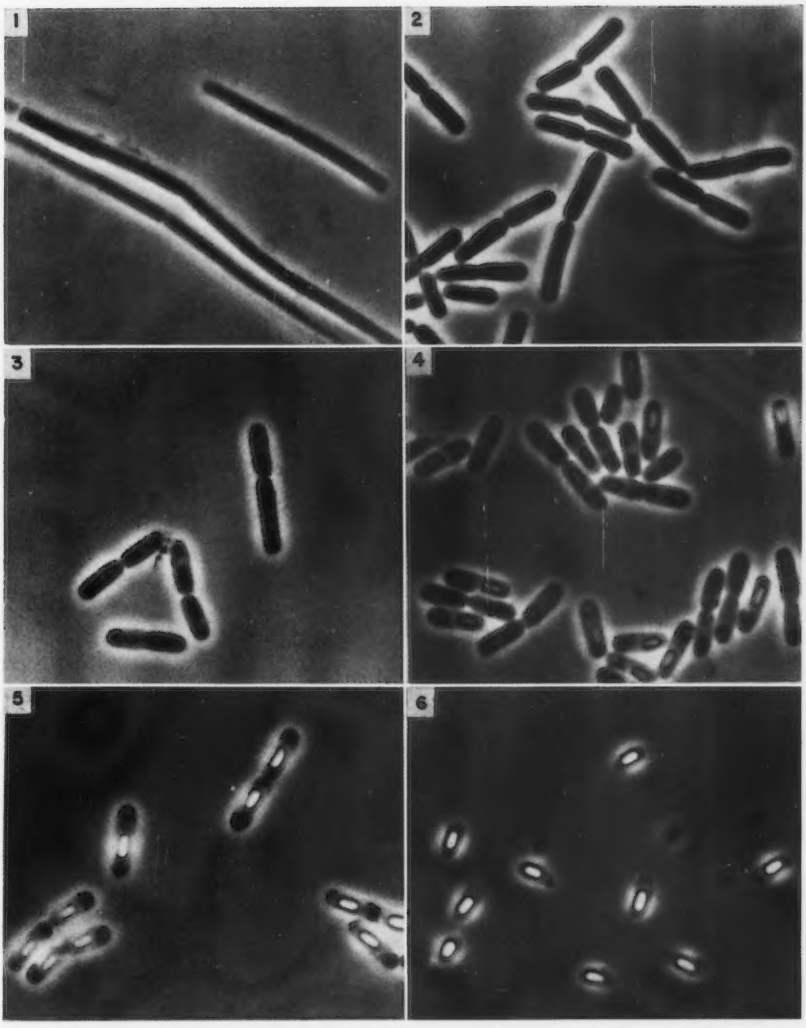
Samples for assay of DPA were quick-frozen in an alcohol – dry ice mixture immediately upon being withdrawn from the carboy culture and held at −40° C. Later, the thawed cells were washed repeatedly in distilled water (analysis of the washes did not indicate any release of DPA) and analyzed by the method of Janssen *et al.* (12). The optical density was measured at 440 mμ with a Beckman DU spectrophotometer. Dipicolinic acid, obtained from Aldrich Chemical Co., Inc., was used as a primary standard.

Viable counts before and after heat treatment were accomplished by making appropriate dilutions in 0.85% (w/v) NaCl and spreading 0.1-ml aliquots on trypticase-soy agar. Heat tolerance was evaluated by exposure at 80° C for 30 minutes of cells diluted tenfold in saline, since under these conditions fully refractile, free spores were found to be resistant while early transitional spores were sensitive (see Fig. 14).

Results

A key requirement for correlating structural and functional changes during sporogenesis is a culture system in which a maturing population undergoes sporulation in a synchronous manner. Such a result fortuitously occurred with the mass culture procedure outlined above and also has been attained by

[5]Recipe for G-medium, modified only in the calcium content from the original (18): 0.4% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.08% $MgSO_4$, 0.01% $MnSO_4.H_2O$, 0.01% $CaCl_2$, 0.001% $CuSO_4.5H_2O$, 0.001% $ZnSO_4$, 0.0001% $FeSO_4$, 0.2% yeast extract, 0.4% glucose. Glucose, phosphate, and calcium solutions are sterilized individually and added to the other components at the time of inoculation.



FIGS. 1 to 6. Stages of sporogenesis in a culture of *Bacillus cereus* strain terminalis recognizable by dark phase contrast microscopy.

FIG. 1. Vegetative cells in the filamentous stage.

FIG. 2. Cells in the granular stage, by which time there is a commitment to sporulation.

FIG. 3. Forespore stage. The cells now occur singly or paired and contain the non-refractile spore and granules at opposite poles.

FIG. 4. Spores in the early transitional stage are but partially refractile, a property which increases in intensity in a given spore and in distribution among the population during this critical stage of development.

FIG. 5. Spores in the late transitional stage are now almost completely refractile in individuals and among the population.

FIG. 6. Free spores after autolysis of the sporangia. The spores are fully refractile and physiologically matured. The exosporium envelope is faintly visible around each spore.

H. O. Halvorson (6), Collier (4), and Fitz-James and Young (5). The degree of synchrony in our cultures may be judged from the observation that virtually complete (>99%) sporulation occurred and that, for example, the time for 50% conversion from forespores to refractile spores was 45 minutes and for 85%, 110 minutes (see Fig. 13). A comparably uniform transition was seen at earlier stages of development as well.

This uniformity and the sequence of recognizable morphological stages that occurred during sporulation of the culture are illustrated in Figs. 1 to 6. Each stage thus identified by phase microscopy could be correlated with the development of anatomical structures observable in thin-sectioned cells by means of the electron microscope, as shown in Figs. 7 to 12, and with some assayable features of physiological maturation, representative and composite data for which are given in Fig. 13. A qualitative profile of the developing spore is given in Table I in the Discussion section. For convenience, the sequence of events will be considered below in each of the recognizable stages; these obviously represent somewhat arbitrary divisions of a gradual cellular change and vary in the corresponding time schedule among experiments.

Filamentous Stage

Under the conditions used, actively multiplying cells of *B. cereus* strain terminalis are filamentous; by phase microscopy, septation is not apparent and the cytoplasm is uniformly homogeneous. Cells in this so-called filamentous stage are shown in Fig. 1. Evidence of differentiation indicative of sporulation similarly could not be discerned in the electron microscope.

Granular Stage

The first microscopic indication that cells are committed to sporulation is a reduction in chaining and a development of granulation. These changes typically ensued about 10 hours after carboy inoculation and the stage persisted without recognizable forespores for 3 to 4 hours. The appearance of cells in this early stage of maturation is shown in the Fig. 2 phase micrograph and the Fig. 7 electron micrograph. In neither is there any evidence of primordial spore structures, although late in this stage the granules migrate to one end of the cell, as seen in the electron micrograph.

A noteworthy physiological change that consistently occurs during this stage is a drop from the usual neutral pH of the culture shortly before observable development of granulation. A pH usually of 5.5 to 5.8 but occasionally as low as pH 5.3 remains for several hours before rising again, at which time clumping of the cells also occurs. Such changes in pH have been observed by others and related to biochemical events during sporulation (6, 14).

However, the most significant feature of this stage is that, despite the absence of spore primordia, cells early in the granular stage have become committed to sporulation. Such granular cells, but not those from the preceding stage, can be washed and transferred to either complete medium or distilled water without interrupting the eventual formation of spores, albeit

DPA-deficient and heat-sensitive ones in the latter case. This observation and its use in determining the nutritional requirement for reversal of the deficiencies are treated in a subsequent paper (1).

Forespore Stage

The discernible shape of an endospore, although not yet refractile and still stainable, marks this stage of development, which usually lasted about 3 hours. As seen with phase optics (Fig. 3), the sporangia occur singly or paired and contain the typically terminal forespores separated from granules arranged largely at the opposite pole.

Electron micrographs of thin-sectioned cells at this stage reveal the beginnings of differentiation in the developing spores. As illustrated in Fig. 8, a single membrane appears first. In time this forespore membrane was observed to thicken and, on the basis of numerous observations, was judged to become the outer coat of the spore.

A homogeneous population of cells in the forespore stage is not yet characterized by any of the physiological properties of mature spores. Such cells did not exhibit any detectable increase in their resistance to heat or phenol, and no trace of DPA could be found by colorimetric analysis (12) or absorption at 270 mμ even after extraction and concentration.

Early Transitional Stage

It is after the appearance of forespores but before the presence of mature spores that outstanding structural and functional changes occur. The approximate time and duration of this and subsequent stages are shown in the upper abscissa of Fig. 13. Early in the transitional stage, the developing spores acquire refractility, a change which is not only quantitative in the population but qualitative in a given spore. That is, both the percentage of refractile spores in a population and the degree of refractility in an individual spore increase. This picture is evident from the Fig. 4 phase micrograph. A decrease in stainable spores occurs almost at the same time and apparently is related reciprocally to the increase in refractile spores, as indicated by the observations plotted in Fig. 13.

Examination of electron micrographs of thin-sectioned cells taken from the first part of the early transitional stage, as illustrated in Fig. 9, revealed further differentiation in the fine structure of the spores. Concurrent with the thickening of the first-appearing outer coat, a second membrane is laid down beside it. This next membrane becomes the exosporium, the outermost

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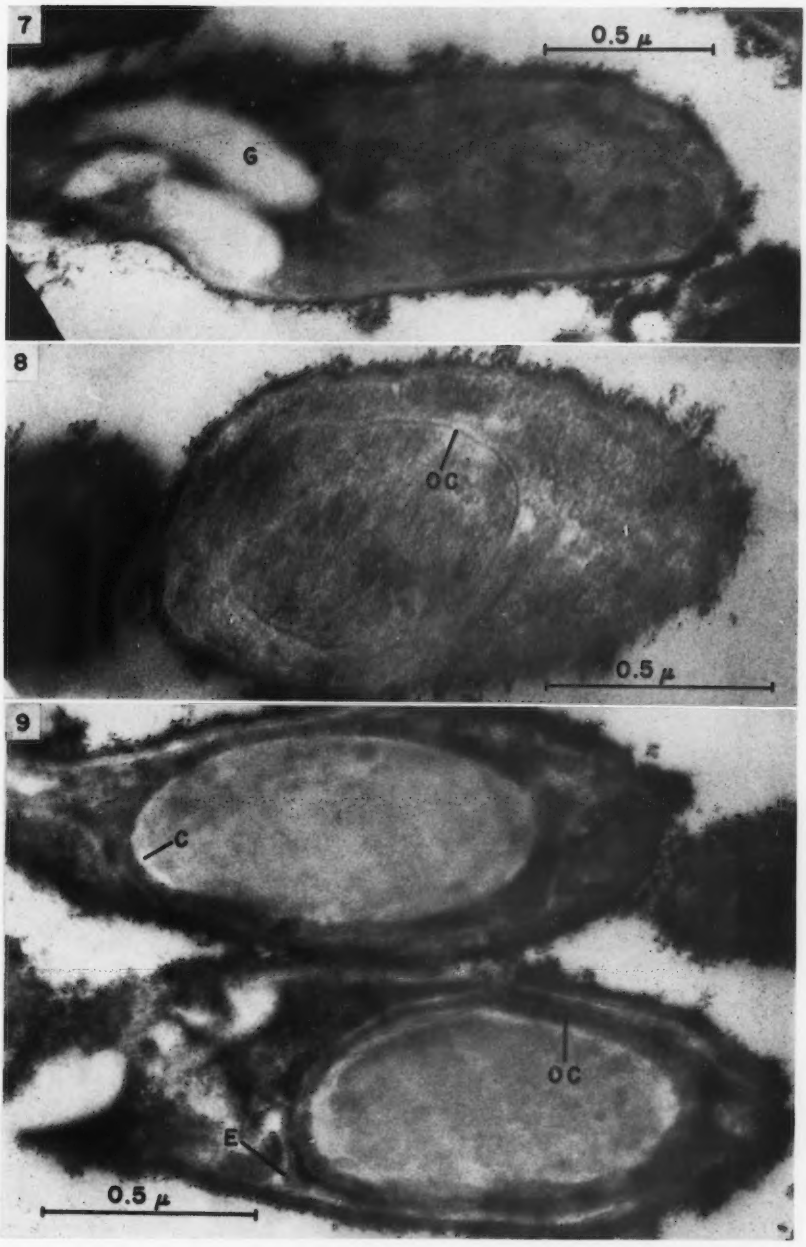
FIGS. 7 to 9. Electron micrographs of ultrathin-sectioned cells representative of progressive stages in sporogenesis.

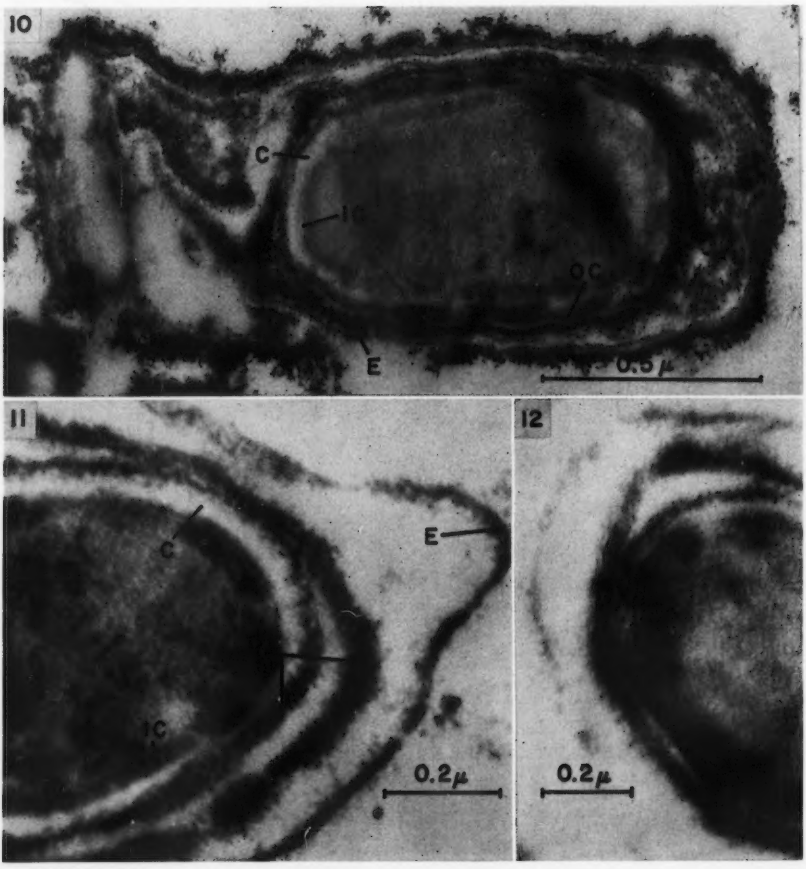
FIG. 7. A cell late in the granular stage, with the granules (G) located terminally but before differentiation of a forespore.

FIG. 8. A sporangium early in the forespore stage. Note the initial appearance of a single membrane (OC), which becomes the outer coat of the matured spore.

FIG. 9. Two sporangia in the first part of the early transitional stage. An exosporium (E) and a primordial cortex (C) have now become evident around the developing spore. The outer coat (OC) has become thickened.



Hashimoto *et al.*—Can. J. Microbiol.



FIGS. 10 to 12. Electron micrographs of ultrathin-sectioned cells representative of stages in spore maturation.

FIG. 10. A sporangium later in the early transitional stage. The exosporium (E), and thickened outer coat (OC), and cortex (C) are evident. Note the faint beginnings of an inner coat (IC) between the cortex and the central core of the maturing spore.

FIG. 11. Part of a free, mature spore prepared and selected to demonstrate the multiple layers of the spore wall: the exosporium (E), split outer coat (OC), cortex (C), and inner coat (IC).

FIG. 12. Part of a similar spore in which lamellation of the outer coat is evident.

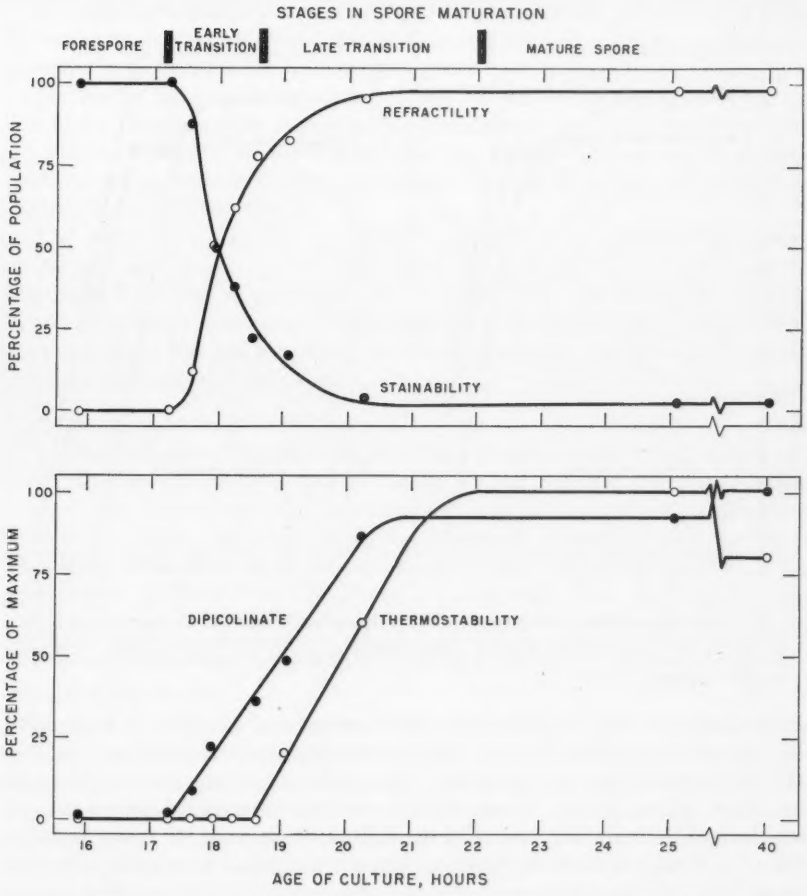


FIG. 13. Some morphological and physiological features which characterize maturation stages of a sporulating population. The actual maximum of DPA synthesized was 89 μg/mg dry spores; each analysis was made on a 4-ml culture sample. The actual maximum count of thermostable spores was 95×10^7/ml.

envelope that characteristically encases spores of the *B. cereus* group. Next to appear is a primordial cortex, an electron-transparent coat that is better seen in the lower cell of Fig. 9. As the cortex develops and thickens through the transitional stages, the inner surface of the cortex, or possibly a separate and new membrane, is formed into a thin inner spore coat (the coat of the core). This last-formed spore coat, seen more clearly in Figs. 10 to 12, will become the cell wall of the newly germinated cell.

As shown in Fig. 13, the early transitional stage is delineated physiologically by the onset of synthesis of DPA *prior to a detectable increase in*

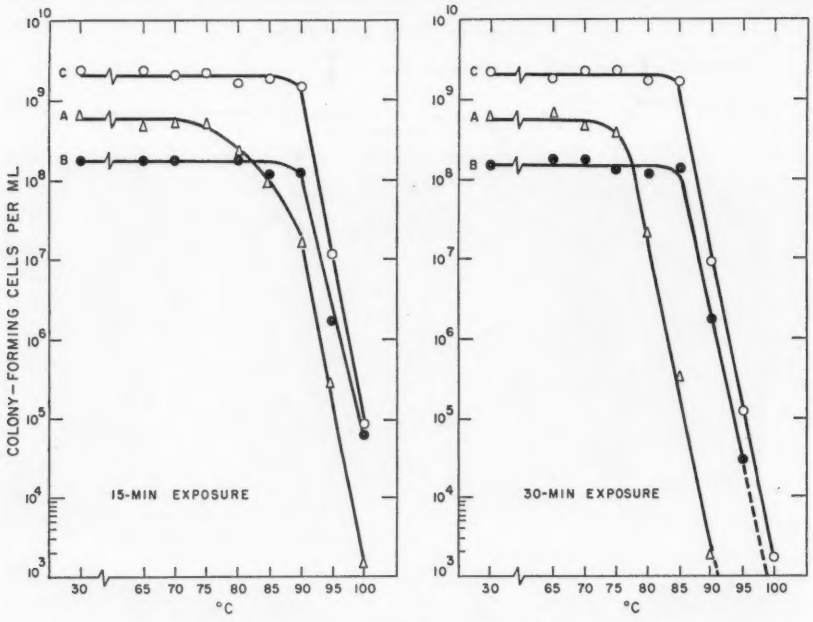


FIG. 14. Comparative heat susceptibility of spores early and late in development. A. Immature spores in early transitional stage, notable for their partial complement of DPA. B. Mature spores after sporangial autolysis. C. Mature spores separated and repeatedly washed in distilled water.

thermostability.[6] This significant observation was confirmed repeatedly. Moreover, it was possible to demonstrate that the spores themselves contained the DPA by sonically disrupting the sporangia and isolating and analyzing the immature spores. It was not uncommon to observe a sporulating culture in which the percentage survival after 30 minutes' exposure at 80° C was less than 1% even though 40% of the maximum level of DPA had been synthesized. At such a time, 75% of the population of spores was at least partially refractile and only slowly stainable.

The selection of time and temperature in determining heat susceptibility is especially important in such a comparison. The choice of 80° C for 30 minutes was not arbitrary but rather came from experiments demonstrating that this amount of heat just begins to kill the immature spores and just fails to kill mature spores. Data comparing the heat susceptibility of a sporulating culture in the early transitional period and late in the maturation

[6]The plotting of dipicolinate and thermostability as dimensionless values in Fig. 13 was done so as to make direct comparison possible at comparable levels of sensitivity. The thermostability curve corresponds to a linear plot of resistant spores such as employed by H. O. Halvorson (6). When a semilogarithmic plot of the spore counts is made, the values plotted essentially as 0 in Fig. 13 are seen to continue as an exponential regression. However, a similar extension of the dipicolinate curve is impossible because of insufficient sensitivity in the analytical method. Consequently, a meaningful correlation between the two characteristics can be made only in the range greater than 1% of the maxima.

stage, after autolysis, are shown in Fig. 14. Two exposure periods and a range of temperature were employed. It can be seen that, with a 30-minute exposure, temperatures below 80° C fail to distinguish heat susceptibility of the spore types; higher temperatures accentuate the differences but do so with a partial kill of the mature spores. A similar but less defined picture can be seen with a 15-minute exposure. In both instances, the shape of the curves is similar, and the resistance of mature spores seems to be unaffected by the presence of culture medium constituents.

The important question of whether the DPA present was distributed uniformly among all, or selectively in a few, spores was not unequivocally resolved. The latter seems less likely, however, since the number of resistant spores counted at low levels of DPA (e.g. 4.6×10^5 spores/ml vs. 10 μg DPA/mg) was 10- to 100-fold less than the number predicted from the usual complement of DPA per resistant spore.

Late Transitional Stage

The second part of the transitional stage is characterized by an increasingly refractile population of spores, as shown in Fig. 13 and the Fig. 5 phase micrograph. The cortex appears to thicken and, at its inner edge, the inner coat of the spore can more clearly be discerned. Physiologically, the spore population gradually acquires heat resistance and, perhaps in parallel, a full complement of DPA (Fig. 13). There is a suggestion from the data that the maximum level of DPA is reached before that of heat resistance, the time for which could not be exactly established from the available data.

Mature Spore Stage

In the final stage of development, the spores are as heat resistant as they will become, have a full complement of DPA, and are fully refractile and stain resistant. Later, the endospores are released by sporangial autolysis. The free spores appear bright and the exosporium is faintly visible by phase contrast (Fig. 6). The small increase in DPA content that seems to occur during autolysis, as shown in Fig. 13, probably reflects only a loss in dry weight on which the DPA content is based. An appreciable decrease in thermostability after autolysis was commonly observed and could not be traced to germination. The change appears to be real and significant, but the basis of it is not apparent.

Some details of the fine structure of the mature spores are revealed in electron micrographs of thin sections. Figure 11 shows part of a formalin-fixed spore in which the multilayered wall structure remains expanded. The exosporium, usually retracted around the spore in the process of embedding, is seen as the outermost envelope. Next under it is the comparatively thick outer coat, which in this preparation has become split. Surrounding the core is an even thicker but electron-transparent region, the cortex. The core itself is encased by the inner coat. These structures are also evident in the Fig. 12 electron micrograph, which additionally reveals a distinctly lamellar substructure of the outer coat.

Discussion

The sequence of recognizable stages that occurs during sporulation of a culture of *B. cereus* strain terminalis has been analyzed separately in the above sections with an attempt to correlate some structural and physiological changes. The main features in the development of a typical spore are qualitatively summarized in Table I.

Characterization of the origin, formation, and substructure of the spore membranes was one objective of the study. Our observations on membrane structure are generally in accord with those published previously (3, 7, 11, 13, 19, 21). Although the number of coat layers is typical, we could not resolve the question of whether the split outer coat is due to the existence of two distinct membranes, an artifact of preparation, or the lamellar substructure.

In less accord with published reports, however, are our findings with respect to the origin and order of formation of the several spore membranes. Chapman (3) has reported that all spore membranes of *B. cereus* appear at the same time, while we and others (19, 21) have observed a sequential development. Furthermore, we have considered that the membranes develop *in situ*, independently of existing sporangial surface membranes. This latter view is not held by Tokuyasu and Yamada (19), who suggest that the cell wall of the sporangium invaginates to form the "spore core wall". Despite careful and repeated search, we have never seen any connection between the cell wall and spore membranes. Moreover, serological studies (20) have shown that isolated spore coats or the surface of spores of *Bacillus megaterium* do not contain antigens in common with the cell wall or other structures of the vegetative cell. Perhaps the most convincing evidence that the sporangial cell wall does not participate directly in spore coat formation follows from the demonstration by Salton (16) that wall-free protoplasts can sporulate. Yet the origination of spore coats from the sporangial cytoplasmic membrane, which we could not observe, cannot be excluded. Fitz-James and Young (6) have noted "a thin vertical septum protruding from the inner surface of the cell wall" which apparently becomes a membrane of the spore primordium; it seems possible that it was a cytoplasmic membrane septum which they observed. Perhaps improved techniques for visualizing the cytoplasmic membrane and for serially sectioning bacteria will help to reconcile these seemingly conflicting views.

TABLE I

Qualitative summary of distinguishing characteristics during sporogenesis

Stage of development	Fine structure	Refractility	Stainability	% of maximum thermo-stability	% of maximum DPA content
Filamentous vegetative cell	Homogeneous	None	Full	0	0
Granular sporangium	Granular	None	Full	0	0
Forespore	Single outer coat	None	Full	0	0
Early transitional spore	Exosporium and primordial cortex	Rapidly increasing	Rapidly decreasing	0	0→40
Late transitional spore	Thickened cortex and inner coat	High	Low	0→100	40→100
Mature spore	Completed differentiation	Full	None	100	100

As another objective of the study, we sought to relate the development of heat resistance, the distinguishing physiological property of mature spores, with the development of fine structure, refractility and stainability, and DPA content. Interrelationships among these other properties were of secondary interest.

The most conspicuous anatomical change that appeared at the time of developing heat resistance was formation of the cortex. This structure first appeared in the early transition stage, before measurable heat resistance but concomitant with the start of DPA synthesis; later the cortex appeared to thicken, and the inner coat to form, as thermostability was acquired. Young (21) previously has observed the association between cortex and DPA formation but unfortunately did not report studies on heat resistance. Maturation of the cortex, and perhaps the inner coat, in relation to the development of both heat resistance and DPA deserves additional study.

Heat resistance also was related to refractility and this, to non-stainability. Both qualitatively (Figs. 1–6) and quantitatively (Fig. 13), the refractility of spores under phase contrast illumination was observed to increase well before the time that thermostability in the population increased. Powell (15) previously has correlated the loss of refractility with a loss of heat resistance in germinating spores and has additionally demonstrated that the heat resistance is lost in the earlier stages of the fall in refractility. Thus refractility seems both to precede the gain and follow the loss of heat resistance. Acquisition of refractility also can be correlated with structural development of the cortex (and possibly the inner coat), as shown previously by Young (21). Refractility and DPA content begin to rise and reach a maximum at about the same time, as noted before (21).

Perhaps the most significant interrelationship observed was that between heat resistance and the DPA content of the population engaged in sporulation. Dipicolinate synthesis was found to begin significantly before a comparably measurable increase in thermostability. Halvorson (6) has reported an even greater disparity in a rapidly developing culture of *Clostridium roseum*, in which the maximum amount of DPA is formed an hour or more before heat-resistant spores can be detected. It would be informative to determine how other species behave in this respect, although comparisons are difficult because of variables in assessing heat resistance (Fig. 14). From the present observations, however, we inferred that a positive correlation between heat resistance and DPA content, if it exists, occurs only above a threshold level of the compound in a developing spore population. An opportunity to check and possibly extend this conclusion to matured spores varying in their DPA content arose in a subsequent study of "endotrophic" sporulation in water (1).

Acknowledgment

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CALCIUM REVERSAL OF THE HEAT SUSCEPTIBILITY AND DIPICOLINATE DEFICIENCY OF SPORES FORMED "ENDOTROPHICALLY" IN WATER[1]

SAMUEL H. BLACK,[2] TADAYO HASHIMOTO,[3] AND PHILIPP GERHARDT

Abstract

Spores of *Bacillus cereus* strain terminalis formed "endotrophically" by transferring granular vegetative cells to distilled water were found to be relatively susceptible to heat and deficient in dipicolinic acid. Calcium ions alone, added in low concentration shortly after the cells were placed in water, could completely relieve these abnormalities. Although the water-formed spores were sensitive to heat, they were as fully resistant as normal spores to gamma radiation or phenol.

Introduction

The heat resistance and dipicolinic acid (DPA) content of spores of *Bacillus cereus* var. *mycoides* reportedly are similar whether the culture is sporulated normally in nutrient solution or "endotrophically" after transfer of the vegetative cells to water (11, 22). These reports suggested to us the possibility of utilizing the technique of "endotrophic" sporulation in water to simplify the preparation of cells for electron microscopy and to facilitate the colorimetric assay for DPA in connection with studies on spore structure and function (12).

Upon analyzing preliminary results with this technique, however, we discovered that water-formed spores of *Bacillus cereus* strain terminalis were relatively susceptibile to heat and deficient in DPA. This unexpected finding posed the question of what exogenous materials are required for the associated properties of heat resistance and dipicolinate content and, more importantly, provided an experimental system to answer it.

Materials and Methods

Bacillus cereus strain terminalis, grown in mass culture in modified G-medium as described previously (12), was used exclusively. Aliquots of the primary carboy culture were removed at appropriate times, centrifuged at 4° C under aseptic conditions, and the cells washed twice by centrifugation in about 40-volume equivalents of sterile distilled water. After an additional wash in either water or the test medium, the cells were resuspended to the original volume and 20-ml aliquots transferred to sterile 250-ml Erlenmeyer flasks. These secondary cultures, which initially contained about 1×10^9 cells/ml, were incubated 24 hours at 30° C on a rotary shaker until completion of the sporulation process.

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Contribution from the Department of Bacteriology, The University of Michigan Medical School, Ann Arbor, Michigan, U.S.A.
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Techniques for assaying dipicolinic acid, evaluating heat resistance, and examining cells under the phase contrast microscope were identical with those previously described (12). Examination of spores in 270 mμ light was accomplished by means of a direct visualizing ultraviolet microscope, made available through the courtesy of Bausch and Lomb Optical Company and Eberbach and Son, Inc.

Results

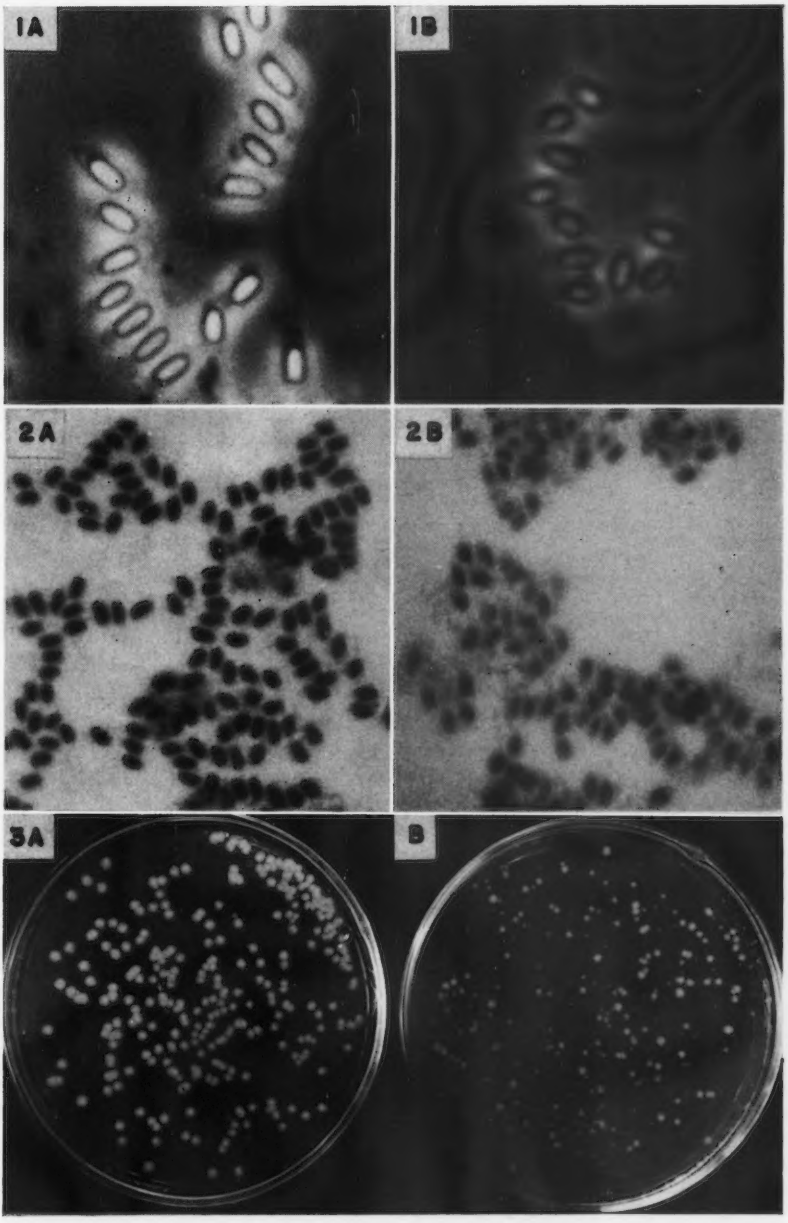
In a preliminary experiment to establish whether the terminalis strain would sporulate in water as reported previously for the *mycoides* variety of *B. cereus*, cells in the filamentous, granular, and forespore stages (12) were taken from a maturing carboy culture, centrifuged, resuspended to original volume in sterile distilled water, and incubated at 30° C overnight on a shaker. Only the granular or forespore cells proceeded to complete sporulation; cells earlier in the growth cycle did not sporulate, consistent with the observation of Nakada *et al.* (18). Water-formed spores were somewhat smaller, rounder, and less refractile than control spores (Fig. 1), but both types were equally resistant to staining. Examination by means of monochromatic (270 mμ) ultraviolet microscopy, as shown in Fig. 2, indicated that the water-formed spores were less absorbing than normal ones.

The incomplete refractility of water-formed spores suggested that these cells, like those from the early transition period in normal development (12), might also be heat sensitive. The validity of this notion was amply demonstrated in the experiment which followed.

In this experiment, the developmental stage of the primary carboy culture was carefully noted, the cells were washed three times before transfer to the secondary sporulation culture, and the population of spores formed in water was characterized quantitatively. The results are given in Table I. Several conclusions are apparent from the data: 1. While the final population consisted entirely (>99%) of viable (>95%) spores in every case, the number of spores formed in water was only 20%[4] (as low as 7% in confirming experiments) of that in the medium control. The loss was attributed to lysis (cf. 20, 23). 2. Varying the time of transfer to water during the 3-hour span from the early granular stage to the late forespore stage did not greatly affect the total population. 3. Although the medium-formed spores contained 78 μg DPA/mg dry spores (a normal complement), the water-formed spores contained an average of only 20 μg of DPA. 4. The spores formed in water were relatively *susceptible* to heat, the kill approximating 99% after 30 minutes' exposure at 80° C. In fact, subsequent experiments showed that even exposure to 60° C for 30 minutes was sufficient to reduce the population by 70%.

While recognizing that the cell lysis attending this technique may actually change the water to a dilute nutrient medium (23), we realized that this so-called "endotrophic" (11) sporulation nonetheless resulted in heat-sensitive

[4]This value indirectly estimates the survival of transferred cells, which could not be accurately counted because of the prevalence of diads and triads.



FIGS. 1 to 3. Comparison of spores formed in control medium (A) and water (B).
FIG. 1. Appearance of washed spores by dark phase contrast microscopy.
FIG. 2. Appearance of washed spores in 270 mμ ultraviolet light.
FIG. 3. Colonies arising from populations of the two spore types.

TABLE I

Heat susceptibility of spores formed in water

Primary carboy culture in G-medium			Secondary sporulation culture					
	% cell type			Analysis 24 hours after transfer				
Hours after inoculation	Granular	Forespore	Medium	% spores	Viable cells/ml before heating	Viable cells/ml after heating*	% survival	μg DPA/mg dry spores
14	100	0	G-medium	>99	9.1×10^8	5.4×10^8	59	78
14	100	0	Water	>99	1.6×10^8	1.9×10^6	1.2	22
16½	98	2	Water	>99	1.6×10^8	2.4×10^6	1.5	17
17½	50	50	Water	>99	1.7×10^8	3.9×10^6	2.3	20
18	0	100	Water	>99	1.6×10^8	3.6×10^6	2.2	23

*In this and subsequent experiments, differential testing was accomplished at 80° C for 30 minutes.

TABLE II

Heat resistance and DPA content of spores formed in water and with selected additives

	Secondary sporulation culture						
		Analysis 24 hours after transfer					
Cell type in primary culture	Medium*	% spores	Viable cells/ml before heating	Viable cells/ml after heating	% survival	μg DPA/mg dry spores
Granular	Water	>99	2.1×10^8	8.2×10^5	0.4	11
	G-salts	>99	8.0×10^8	5.3×10^8	66	51
	G-salts+alanine+pyruvate+aspartate	>99†	9.0×10^8	2.9×10^8	32	44
	G-salts+glutamate+pyruvate+aspartate	< 1				
Forespore	Water	>99	1.7×10^8	3.3×10^6	1.9	10
	G-salts	>99	9.4×10^8	4.8×10^8	51	46
	G-salts+alanine+pyruvate+aspartate	>99	1.1×10^9	3.9×10^8	35	40
	G-salts+glutamate+pyruvate+aspartate	< 1				

*"G-salts" refers to the modified G-medium without glucose or yeast extract. Twelve μmoles of each organic acid were added to 20 ml of medium.
†Forty per cent of the spores in this culture were germinated but not grown out.

and DPA-deficient spores and so might provide a means to determine the exogenous nutritional requirements for restoring these properties.

The next several experiments, therefore, were designed to determine what component or components of G-medium could be substituted for the complete medium in reversing the heat susceptibility and dipicolinate deficiency of spores formed "endotrophically." The experiment recorded in Table II employed the inorganic salts of G-medium as a basal solution, with the initial idea of alleviating the lysis that occurred when cells were transferred to water alone. These salts, together with possible DPA precursors (17), were added to the secondary sporulation suspension. To be sure, the balanced salt mixture decreased the lysis with a resulting increase in the number of spores formed. But more importantly, the G-salts alone also induced a marked increase in both the heat tolerance and DPA content of the spores. The amino acids or pyruvate added as possible DPA precursors actually lessened the inorganic salt effect on DPA synthesis and heat resistance, and glutamate even inhibited sporulation.

Attention was then directed to determining the active component(s) of the G-salts. Curran *et al.* (5) and others (8, 9, 10, 28) have long recognized that

calcium must be included in complex growth media for the development of thermostabile spores. Others have reported that calcium may be present in such spores as the salt of dipicolinic acid (19, 24, 34). Consequently, it seemed reasonable to suppose that calcium by itself might induce heat resistance and DPA in spores forming in water. Furthermore, it seemed possible that manganese, whose role in sporulation has also been emphasized (1, 3, 6, 8, 30), might be active in the synthesis of DPA, substitute for calcium in the production of heat-resistant spores, or protect the primary granular cells from lysis.

A typical experiment designed to test these possibilities is summarized in Table III. It is notable that, in every case where calcium was present, not only was the heat-survival percentage high but also the DPA content was high. Conversely, in every case where calcium was lacking, both the heat tolerance and the DPA analysis were low. In fact, it appeared from this and subsequent confirmatory experiments that spores formed in water plus calcium show a *higher* heat-survival ratio than spores formed in G-salts or even in complete G-medium. In contrast, manganese only slightly increased the percentage of heat-resistant spores, although these contained 30 μg DPA/ml. This small effect of manganese may have been due to contamination with calcium ions. (In another experiment, cells grown either in complete G-medium or in G-medium in which the calcium was replaced with equimolar strontium were transferred in the granular stage to strontium solutions. The spores in both cases showed about 30% survival to heat, and the DPA content was 88 and 75 μg/mg dry spores, respectively.)

These experiments (Table III) also gave some interesting information on cell lysis in the secondary cultures. The addition of calcium alone to the water reduced the amount of lysis, but this effect was not seen with manganese. The withdrawal of either calcium or manganese from G-salts in the secondary

TABLE III

Heat resistance and DPA content of spores formed in water with inorganic additives*

Secondary sporulation medium†	Analysis 24 hours after transfer				
	% spores	Viable cells/ml before heating	Viable cells/ml after heating	% survival	μg DPA/mg dry spores
Water	>99	1.6×10^8	2.8×10^6	1.7	16
G-salts	>99	9.6×10^8	5.3×10^8	55	63
G-salts less Ca^{++}	>99	1.6×10^8	2.2×10^6	1.4	18
G-salts less Mn^{++}	>99	5.5×10^8	2.7×10^8	49	70
G-salts less Ca^{++} and Mn^{++}	>99	$<10^6$	$<10^4$	1	0
Water+Ca^{++}	>99	4.7×10^8	5.3×10^8	113	59
Water+Mn^{++}	>99	2.2×10^8	9.7×10^6	4.5	30
Water+Ca^{++}+Mn^{++}	>99	3.0×10^8	3.0×10^8	100	62
Water+NaCl	>99	3.3×10^8	7.5×10^5	0.2	14

*In this and subsequent experiments, the primary culture at time of transfer consisted entirely of granulated cells.
†Ca^{++} and Mn^{++} were added in the same species and concentration as in the modified G-medium. NaCl was added at 0.85% (w/v).

sporulation medium consistently increased the lysis. If both were removed, the lysis increased still more so that less than 0.1% of the transferred cells survived. Population stabilization does not seem to be a simple osmotic phenomenon, since cells transferred to saline underwent the same degree of lysis as those transferred to water. In other experiments, it was found that neither any other single salt of the G-salt mixture in an equivalent concentration, nor 10% Carbowax 4000, contributed to population stability. However, granular cells transferred to a solution consisting of 0.2 *M* sucrose, 0.02 *M* maleate buffer at pH 7, and calcium gave a spore population which was 70% of that in the G-medium control and exhibited comparable heat resistance and DPA content.

The primary finding that the addition of calcium alone could induce a maximum degree of heat resistance and a near-maximum synthesis of DPA during "endotrophic" sporulation prompted us to characterize the calcium effect more exactly. Table IV summarizes the results of an experiment in which the concentration of calcium chloride added to "endotrophically" sporulating cells was varied between 0 and 1000 mg/liter. The significant finding in this experiment was that, when the concentration of added calcium chloride was less than 20 mg/liter, not only did the heat tolerance of the spores decline but also the DPA content fell to a level comparable with that of spores formed in distilled water. Since the calcium level necessary to reverse the heat susceptibility and DPA deficiency of water-formed spores appeared to be low, but critical, it seemed possible that this requirement might also be met by growing the cells initially in a medium rich in calcium. This proved to be partially true. Cells grown to the granular stage in G-medium with 1000 mg calcium/liter and then washed and transferred to distilled water yielded nearly 100 times more heat-resistant spores than usually found, and the DPA content was

TABLE IV

Effect of calcium concentration on heat resistance and DPA content of spores formed in water solution

Mg $CaCl_2$/liter secondary sporulation solution	Analysis 24 hours after transfer				
	% spores	Viable cells/ml before heating	Viable cells/ml after heating	% survival	μg DPA/mg dry spores
0	>99	1.2×10^8	5.4×10^5	0.45	14
0.1	>99	1.1×10^8	6.0×10^5	0.55	17
1	>99	1.4×10^8	1.7×10^6	1.2	19
10	>99	2.3×10^8	5.7×10^7	25	41
20	>99	2.5×10^8	2.3×10^8	92	57
50	>99	5.2×10^8	4.3×10^8	83	62
100	>99	5.6×10^8	4.8×10^8	86	75
1000	>99	7.4×10^8	4.6×10^8	62	72
G-medium control	>99	1.3×10^9	7.5×10^8	58	75
G-salts control	>99	7.6×10^8	3.1×10^8	41	44

twice that obtained when cells were transferred from the normal amount of calcium. Thus, enough calcium apparently accumulated during vegetative growth to obviate the requirement for additional calcium later during sporulation. Alternatively, an adequate calcium level may have been provided from the lysis of calcium-rich cells.

The foregoing results, together with those of Vinter (29) which show a marked effect on calcium accumulation with the time of addition of cystine to a sporulating culture, led us to believe that there might be a direct dependence of heat susceptibility and DPA content of water-formed spores on the time of addition of calcium to the secondary sporulation culture. The results from a typical experiment examining this point are indicated in Table V. The addition of calcium to a primary culture in the granular stage was more effective than in the forespore stage. Subsequent experiments have confirmed our impression that calcium must be available before the early transition stage; that is, calcium is without effect when added to water-formed spores that are already partially refractile.

TABLE V

Effect of time of calcium addition on heat resistance and DPA content of spores formed in water solutions

Time of Ca^{++} addition after transfer to water,* hours	% cell type		Analysis 24 hours after transfer			
	When Ca^{++} added	Final	Viable cells/ml before heating	Viable cells/ml after heating	% survival	μg DPA/mg dry spores
0	100 granular	>99 spores	2.3×10^8	1.8×10^8	78	50
1¼	100 granular	>99 spores	1.4×10^8	1.1×10^8	79	57
3½	95 granular	>99 spores	2.4×10^8	1.4×10^8	58	56
4½	10 forespores	>99 spores	2.2×10^8	1.3×10^8	59	40
6¾	100 forespores	>99 spores	1.6×10^8	8.0×10^7	50	48
7¾	20 spores	>99 spores	1.8×10^8	8.3×10^7	46	42
8¾	30 spores	>99 spores	1.7×10^8	9.7×10^7	57	42

*$CaCl_2$ was added at a final concentration of 100 mg/liter in each case.

Attention was next directed to characterizing further the thermolabile, DPA-deficient spores that form in distilled water. When normal, mature spores are placed in trypticase-soy broth supplemented with L-alanine and adenosine and are aerated by shaking, complete germination occurs within 5 minutes. If water-formed spores are similarly treated, germination was found to proceed much more slowly and to require several hours for completion. It was also noted that colonies grown from water-formed spores appear abnormal. Plates of colonies from normal and water-formed spores are contrasted in Fig. 3. The colonies of the deficient spores are heterogeneous in size and the small colonies never reach the size of normal colonies even after prolonged incubation. These observations suggest that not only the germination rate of the spores but perhaps also the generation time of the vegetative progeny has been affected.

Another point of interest in characterizing water-formed spores was the distribution of heat susceptibility in the population. The fact that a small but measurable percentage of such spores survived 80° C for 30 minutes indicated a heterogeneous population. The bimodal inactivation curve obtained with these spores (Fig. 4) was consistent with this idea. The initial rate of kill ($K=0.67$) is about what might be expected for complete susceptibility, as in vegetative cells. The secondary rate ($K=0.0020$), however, is still greater than that ($K=0.0030$) observed for the control spores, although this difference could be an artifact. If the heat susceptibility of the spores were evaluated in terms of the initial inactivation rate rather than the percentage of survivors, the relative heat susceptibility of water-formed spores would, of course, seem even more dramatic.

Although heat resistance of spores has long been inferentially associated with the presence of DPA, these properties have not been related to spore

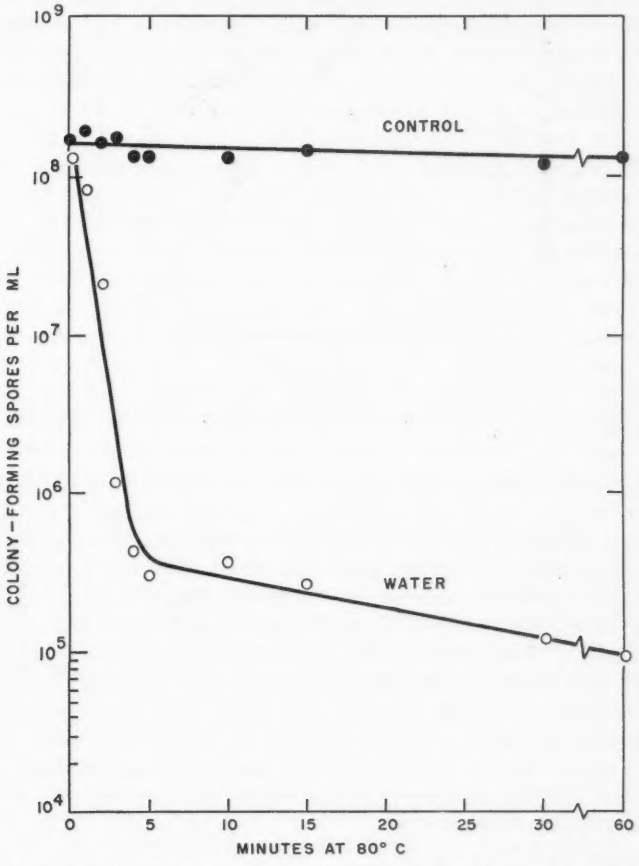


FIG. 4. Heat inactivation rates of spores formed in water and in control medium.

resistance to other agents. Accordingly, spores formed in distilled water were compared with normal spores for their resistance to other agents. Gamma radiation was initially tested. Normal and water-formed spores were suspended in 0.9% (w/v) sodium chloride solution to a concentration of about 10^6 spores/ml and exposed for various lengths of time to a cobalt-60 source. Replicate vials were removed at intervals and the number of colony-forming spores counted on trypticase-soy agar. The results shown graphically in Fig. 5 clearly indicate that the DPA-deficient spores were in fact *less* susceptible to gamma radiation than the normal spores. A similar observation under different conditions has been noted by Woese (32), who found that from the time

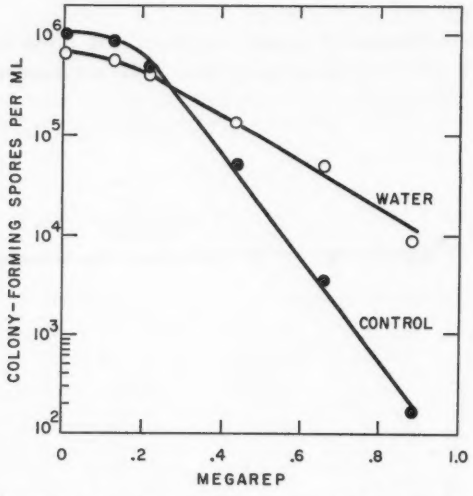


FIG. 5. Comparative resistance of spores formed in water and in control medium to gamma radiation from cobalt-60.

when spores of *B. megaterium* release their dipicolinic acid until about an hour later in germination, there is only a negligible change in sensitivity to ionizing or ultraviolet radiation. Woese's (31) conclusion that radiation damage to bacterial spores is genetic also was indirectly confirmed by our experiments. Spores exposed to 0.8 megarep[5] or even to 8.0 megarep were able to germinate and release DPA when transferred to the proper medium but were not able to divide. A similar observation has been reported by Hermier (14) also employing cobalt-60 radiation, by Robinow (25) using X rays, and by Levinson and Hyatt (16) using the ionizing electron beam from a Van de Graaff accelerator. This apparent divorcement of germination from subsequent outgrowth may provide a useful means for studying the underlying mechanisms of spore germination and has obvious practical implications in the use of ionizing radiations to preserve food.

[5]rep = roentgen equivalent physical.

Apparently the normal or even greater resistance of the DPA-deficient, heat-susceptible spores is not limited to ionizing radiation. In a similar comparison of phenol resistance, both normal and water-formed spores were equally resistant to 3% phenol solution over a week's exposure. It appears, therefore, that the mechanism of spore resistance to different deleterious agents not only differs but that such resistance also may or may not be correlated with the DPA content of spores.

Conclusions and Discussion

The results justify three principal conclusions: 1. Spore populations of *B. cereus* strain terminalis formed "endotrophically" by transferring granular vegetative cells to water are relatively heat-susceptible and DPA-deficient. 2. Calcium ions alone, added early in sporogenesis, can completely relieve these abnormalities. 3. Although the water-formed spores are susceptible to heat, they are as fully resistant as normal spores to gamma radiation or phenol.

The phenomenon of vegetative cells sporulating after transfer to water has repeatedly been observed in the past (2, 15, 18, 26). But in none of these cases were spores assayed for chemical components or heat resistance. However, Hardwick and Foster (11) and Perry and Foster (22) have published experiments which indicated that such "endotrophically" formed spores of *B. cereus* var. *mycoides* were normal in both their heat resistance and their DPA content. Our experiments with *B. cereus* strain terminalis, reported above, gave quite the opposite results. Their test organism was, of course, different. Another point of difference was the concentration of vegetative cells transferred to water; while we routinely used 10^9 cells/ml, Perry and Foster (20) used 10^6 cells/ml. It may be informative that a comparable concentration (ca. 10^6) of the spores formed in our system displayed a normal degree of heat resistance, although these represented only about 1% of the total spores. Since the heat resistance and DPA content were shown to be quantitatively related to the concentration of calcium present during sporulation, it is conceivable that the residual heat-resistant spores in our system and most of this type in their system reflected the calcium available from chance contamination or from cell lysis.

The latter possibility raises again (cf. 20, 23) the question of whether "endotrophic" sporulation is, in fact, dependent on nutrients released by autolysis of part of the vegetative population after transfer to water. Lysis did indeed occur in our system, so that the water was probably changed to a dilute nutrient medium. Yet this environment must be deficient, since the spores formed were abnormal and did not subsequently germinate or recycle into vegetative cells. Regardless of whether "endotrophic" sporulation occurs in water or actually in dilute medium, the system provided a means of producing heat-sensitive, DPA-deficient spores and of determining specific nutritional requirements to relieve the abnormalities. We have preferred to qualify the term "endotrophic" by the use of quotation marks in describing the sporulation that occurs when vegetative cells are transferred to distilled water.

Although it has been shown by others that calcium is necessary for the formation of heat-resistant spores, our experiments using the "endotrophic" system additionally demonstrated that calcium alone can direct the synthesis of DPA in conjunction with the acquisition of heat resistance. Calcium was seen to relieve also the morphological abnormalities which have been described previously by Grelet (7) for *B. megaterium* sporulated in complex medium deficient in calcium. The requirement for calcium may well account for other situations in which abnormal spores are formed. Vinter (29) has reported that the addition of cystine to sporulating cultures of *B. megaterium* results in "defective spores". He has suggested that "probably one of the most important reactions occurring during spore formation, that concerning calcium accumulation, i.e. the synthesis of dipicolinic acid, is disturbed." Young (33) has reported a similar effect for cysteine and found also that versene added to a sporulating culture of *B. cereus* prevents complete refractility and the synthesis of the full amount of DPA in the spores formed. Presumably the mechanism of action in this case is one of chelation of divalent cations, including calcium, present in the sporulating medium.

Calcium might be involved in reactions leading to the synthesis of DPA, either from endogenous or external precursors, or in reactions trapping the product. The latter possibility should permit DPA synthesis to occur "endotrophically" in a calcium-deficient system, the DPA accumulating in the medium; yet analyses of the water after sporulation negated not only this but also the possibility that partial germination was occurring. Further evidence against a simple trapping function for calcium is found in reports that the molarity of calcium in spores is considerably less than that of DPA (21, 27). Rather, it appears that DPA synthesis occurs from endogenous precursors late in sporogenesis, since the requirement for externally supplied calcium must be met before the initiation of cortex development and loss of internal stainability, that is, conceivably before the spore core becomes impermeable. Where the DPA is located in the spore has not yet been determined. On one hand, the spore region that apparently corresponds to the cortex was not seen to absorb a band of ultraviolet light specific for DPA (13), indicating that DPA probably is not localized in the cortex per se. On the other hand, conditions of growth which prevent the synthesis of a full complement of DPA in spores also prevent complete development of the cortex (33).

Recently Church and Halvorson (4) have reported a quantitative relationship between heat resistance and dipicolinate content in spores of *B. cereus* strain terminalis made deficient in DPA by changes in the growth medium. In view of our similar conclusion with the "endotrophic" system, it seemed desirable to compile the results accumulated during this study into a plot analogous to theirs of the mean inactivation rate (30 minutes at 80° C) vs. the DPA concentration in the spores. Our use of a mean determination between two points allows, of course, only an approximation of the rate and inaccurately represents situations where the inactivation curve is bimodal, as in Fig. 4. Yet within these limitations, the results shown in Fig. 6 provide several notable points. Above 50 μg DPA/mg dry spores, the heat suscepti-

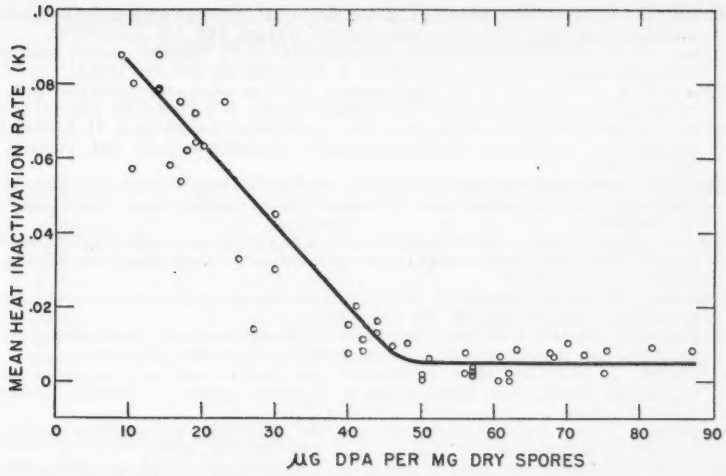


FIG. 6. Correlation between dipicolinic acid content and the mean heat-inactivation rate (K) of spores formed in various secondary media. K was calculated from the equation, $K=(1/(t_2-t_1))\log(N_1/N_2)$, where the survival ratio was determined from an initial count and another after a 30-minute exposure at 80° C.

bility of the population appears to remain relatively constant and minimal. Below the 50-μg level, the rate of heat inactivation increases rapidly and in proportion to the amount of DPA. In confirmation of these results with "endotrophic" spores, our data on DPA content and heat resistance during normal sporogenesis (Fig. 13 in ref. 12) also were found to be consistent with the bimodal curve of Fig. 6. Church and Halvorson (4), too, have pictured a bimodal curve but with significant quantitative differences. First, the rates of inactivation they have recorded for resistant spores generally are high, even when recalculated to a minute basis (correction by private communication from Halvorson). Second, the break in their curve occurs at a comparatively lower level of DPA. Third, their inactivation rates at higher levels of DPA continue to decrease with increasing DPA content, in contrast with the plateau we observed. We have been unable to reconcile these discrepancies.

Acknowledgment

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A HETEROTHALLIC, INOPERCULATE DISCOMYCETE: MORPHOLOGY AND DEVELOPMENT[1]

R. H. HASKINS

Abstract

Studies of the fungus which produced large quantities of the recently described pigment, mycochrysone, revealed minute white apothecia which possessed a hymenial layer of slender paraphyses and inoperculate eight-spored asci. The eight ascospores of a single ascus produced four black (−) and four brown (+) colonies. Apothecia were formed in vitro, initially only along the lines of junction of these two readily distinguishable types of colonies. Phialospores and pycnidia-like structures were produced by the black (−) strain. Mycochrysone was produced only by the brown (+) strain. The various fruiting structures are described and illustrated. The organism could be a *Calloria* of the Helotiaceae but positive identification is not yet possible.

Introduction

During studies on metabolic products of various fungi, a culture, received in 1953,[2] of an unidentified organism isolated from a diseased *Pinus sylvestris* seedling, was examined. This organism, when grown on Difco potato dextrose agar, was found to produce large quantities of extracellular bright orange-red crystals, both in the agar medium and in the exudate on the surface of the culture. Preliminary chemical studies on this crystalline material, named mycochrysone, have been reported elsewhere (7).

Eventually, several apothecia appeared on the surface of an old culture. Dissection of a portion of one of the apothecia showed it to possess a hymenial layer of slender paraphyses and inoperculate eight-spored asci. The plate containing the remaining apothecia was inverted over a fresh potato dextrose agar plate and left in that position overnight.

By the next morning many ascospores had been discharged onto the surface of the agar below, where they had germinated. Small blocks of agar, each bearing a single germinating ascospore, were cut, aseptically, from the edges of the areas showered with spores, and were placed on fresh potato dextrose agar plates, where the germinated spores continued development as single-ascospore cultures.

The single-ascospore cultures that developed were of two distinct types (Figs. 1–3). One type was smooth, black, and often shiny, spreading thinly but compactly over the surface of the agar, but penetrating it with colorless hyphae. The other type was cottony, with abundant aerial hyphae, cinnamon brown in color, with similarly colored mycelium penetrating into the agar into which a brownish-orange pigment diffused. It was only by this second type of colony that the orange crystals were produced.

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Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Presented, in part, before the Botanical and Mycological Societies of America at Storrs, Connecticut, 1956.
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[2]Dr. Olli Vaartaja, Forest Pathology Laboratory, Saskatoon, Saskatchewan. Original No. 601, collected at Indian Head, Saskatchewan.

By inoculating agar plates with the two types of single-ascospore cultures, it was found that apothecia were produced, initially, only at the lines of junction of the two types of colonies (Figs. 4–6). About 1200 crosses have been made involving 64 brown (+) single-ascospore cultures and 35 black (−) single-ascospore cultures, with fertile apothecia being formed, initially, only along the line of junction of a brown (+) and a black (−) colony. Apparently the plus and minus characteristics of this fungus are closely linked to those of the colony types of brown and black. Not often are the + and − strains of a heterothallic (dioecious) organism so distinct so early in colony development.

Taxonomically, the organism could be a *Calloria* of the family Helotiaceae (5, 8) but full and accurate identification of the fungus will have to wait, probably, until it is found again in nature, in its sexual fruiting stages.

Repeated inoculations of *Pinus sylvestris* have so far been unsuccessful. Having examined a culture bearing apothecia, Dr. J. W. Groves (Plant Research Institute, Canada Department of Agriculture, Ottawa) (5) expressed the opinion that it might be a *Karschia* or a *Calloria*, though *Karschia* species have larger and more ellipsoid spores and are hard in consistency, and *Calloria* does not seem to occur on pine. Comments, advice, etc. from the reader will be welcomed.

For convenience, in the descriptions that follow the strain producing the black type colony will be termed "black (−)" and the other strain "brown (+)", without necessarily implying any maleness or femaleness to either one or the other.

Colony Characteristics

Black (−) Strain

The surface of the colony (Figs. 1–4) is at first black, smooth and shiny, later often becoming greyish-black, dull matte in texture with occasional dark arbusculate tufts of sparse aerial mycelium or with a more or less complete cover of low, dense, dark-grey aerial mycelium. Occasionally, radiating low ridges are formed. The margin is entire, and there is a narrow band (1–2 mm) of white mycelium at the advancing edge (Figs. 1–2). The black mycelium is limited to a thin, tough, compacted layer along the surface of the agar (Figs. 1–2 inserts, 38). The hyphae penetrating into the agar are relatively sparse and are colorless, contributing to the greyish-white color of the reverse side of the colony. The mycelium is regularly septate, 2.5–3.5 μ in diameter, with color, where present, in the cell walls. Interhyphal anastomoses are frequent.

In mature colonies, black sclerotia or pycnidia-like bodies (10–200 μ in diameter) may appear (Figs. 36–38), at first singly, later in groups and clusters, often confluent with one another. These bodies appear in most single-ascospore cultures of this black (−) strain, and especially where such colonies are adjacent to the advancing edge of a brown (+) colony. Initially the bodies are light in color, soon becoming very dark brown to black because of pigmentation in the walls of the outer layers of hyphae (Figs. 36–38). The contents remain thin-walled and hyaline.

Brown (+) Strain

This strain produces, from the first, rather abundant cinnamon or reddish-brown arbusculate aerial mycelium (Figs. 1–4). The margin of the colony is entire and shows a 1- to 2-mm zone of white mycelium, both at the agar surface and in the aerial mycelium at the periphery of the colony (Figs. 1–2). As the colony develops, bright orange crystals often appear along the aerial hyphae, and particularly beautifully in the rather abundant exudate near the center of the colony. In some cultures these crystals appear along the surface of the agar and, more yellow in color, in the medium to the full depth of the agar.

The mycelium extending into the agar (Figs. 1–2 inserts) is colored brown and a pigment diffuses from it into the agar, eventually coloring the whole agar plate. The pigment is an indicator which turns from its orange-brown to purple with the addition of alkali. In mature and old plate cultures, the agar often changes from an orange-brown color, through a purple, to a dark brown which is intensified considerably by the desiccation of the agar. The color of the reverse of a growing culture is reddish-brown to dark brown.

The mycelium is regularly septate, 2.5–3.5 μ in diameter, freely branching with frequent anastomoses.

In many cultures, tufts of white aerial mycelium (apothecial initials?) appear on the surface of the colony, particularly towards its periphery. These tufts are particularly prominent along the line of junction between colonies of the opposite strain, and are described more fully below.

Mixed Cultures of (+) and (−) Strains

The appearance of mixed colonies depends on several factors. Cultures from very light but widespread spore discharge show patches typical of each strain as well as patches unlike either. When equal portions of pure cultures of each strain were blended in sterile distilled water and the resulting suspension used to inoculate potato dextrose agar plates, more or less greyish-black colonies resulted which eventually produced scattered apothecia. Generally, the orange crystal production was not evident unless the mixed inoculum contained a large percentage of the brown (+) strain.

Fruiting Structures

Apothecia

The apothecia (Figs. 4–6, 10–15) are essentially sessile, white to greyish-white in color, solitary or gregarious, up to 1 mm in diameter. They are a glistening white when young, becoming dull greyish and finally dark brown when overmature.

The first signs of developing apothecia are the white tufts of mycelium (Figs. 5 and 10) at the surface of the agar or amidst the aerial mycelia at the junction of a brown (+) and a black (−) colony. Such tufts appear towards the periphery of brown (+) colonies, but do not normally form mature apothecia in the absence of the black (−) strain.

These white tufts are made up of short hyaline hyphae with swollen ends and often have the appearance of receptive hyphae with trichogynes (Fig. 10). Various apothecia in different stages of development are shown in Figs. 10–15. At no time does the hymenium seem to be covered with an exciple. The asci are exposed from the first.

Asci and Paraphyses

The ascus is more or less cylindrical (Figs. 16–19), and is inoperculate. Its walls are thickened at the distal end and there is a definite exit pore or channel (Fig. 20). It averages 4×40 μ in size, with the spores at maturity occupying the distal half of the ascus. The spores in undisturbed asci are usually obliquely diseriate becoming obliquely uniseriate. Crozier-hook formation was readily found in dissected material but was difficult to photograph clearly (Figs. 16–19).

The paraphyses are slender and generally unbranched (Fig. 17).

Ascospores

The ascospores (Figs. 18–19, 21–22) averaged 2×8 μ in size and were aseptate becoming one-septate usually after discharge, but, occasionally in a long undischarged ascus, the spores became enlarged, thick walled, brown in color, definitely one-septate (Fig. 15), and somewhat constricted at the septum. The spores were often found adhering in groups to the aerial hyphae (Fig. 21).

The spores germinated readily producing one, two, or more germination tubes (Figs. 7–9). As already mentioned, the ascospores produce two obviously different types of colonies. Using a De Fonbrune micromanipulator, a single mature ascus was isolated from an apothecium. The eight ascospores from this single ascus were separated and eight single ascospore cultures were obtained. Four of these were of the brown (+) type and four of the black (−) type. The eight cultures were inoculated into a single potato dextrose agar plate to give the colonies as shown in Fig. 3. Apothecia were produced initially only along the lines of junction of the two types of colonies (Figs. 4–6). To complete the classical experiment, the four distal ascospores were isolated, in sequence, from another ascus. These on germination produced two brown (+) and two black (−) colonies, showing that the segregation of the colony types took place, in this case, during the second division of the diploid nucleus.

Pycnidia

The function of the black sclerotia or pycnidia-like bodies (10–200 μ in diameter) (Figs. 36–38) has not been determined. They are produced only by the black (−) strain and consist of an outer layer of darkly colored, interwoven relatively thick-walled, sclerenchyma-like hyphae, and an internal mass of thin-walled parenchyma-like "tissue". The function of the microconidia- or spermatia-like cells (1.3×4–6 μ) obtained occasionally by crushing these bodies is not known.

Conidiospores

An indication of conidiospore-like cells produced on phialides was first observed when the mycelium of a black (−) culture grew across the exposed glass bottom of a petri plate culture from where a block of agar had been removed. Examination of the low, dense, dark-grey aerial mycelium, produced in some cultures of this strain, revealed endoconidiospore or phialospore formation (Figs. 23–35). The first-formed spore is elongated (Figs. 27 and 35 (lower right)). After its release the collarette becomes evident (Figs. 24 and 31). Subsequent spores are more or less oval to subspherical in shape, 1.3–2.5 μ in diameter (Figs. 23 and 35). A series of developing spores was often evident in the phialides, particularly in the terminal ones (Figs. 25, 30, and 32). The discharged spores, initially in a single chain (Figs. 29 and 33), gathered in sticky masses around the tip of the phialide (Figs. 25, 26, 28, and 34). In many cases, the distal end of the phialide was bent back (Figs. 27, 29, 31, and 33) so that the collarette and the discharged spores were directed back towards the phialide proper (Figs. 29 and 33). Proliferation of the phialide occurred from the cell immediately below the collarette (Fig. 33) rather than through the collarette. The phialide may be terminal and elongate (Figs. 23–33) or lateral, short, and flask-shaped (Fig. 34).

These endogenously produced spores (Fig. 35) were spread over the surface of potato dextrose agar and incubated at room temperature. Germination of the spores was not observed during 2 weeks of incubation, by the end of which time growth from mycelial fragments had obscured the spores. It is possible that these spores may function as spermatia.

Metabolic Products

Two metabolic products are presently under study. Preliminary chemical studies (7) on the orange-red crystalline pigment "mycochrysone" have already been mentioned. This pigment is produced in yields of over 4% of the total dry weight of mycelium plus agar. Yields on shaken liquid cultures were not appreciable.

As already reported (7), mycochrysone shows antibiotic properties in common with other quinone compounds. Tests to date have not shown anything particularly startling, though its antifungal abilities may prove of value. Other discomycetous fungi have been shown to produce quinones with antifungal properties (4, 6).

During the isolation and treatment of over 90 single ascospore cultures from ascospores developed in apothecia produced by crossing brown (+) and black (−) cultures which had been conserved under sterile mineral oil (2), isolates of the black (−) type were obtained which produced large amounts of viscous liquid. So abundant was this production that in inverted agar plate cultures the liquid dripped from the culture onto the cover of the petri plate below. Preliminary investigation showed this material to be polysaccharide in nature. Shaken liquid cultures are being used to produce amounts adequate for further chemical study.

Discussion

The number of ascomycetes known to have a heterothallic development similar to the present fungus is increasing. *Ascobolus geophilus* Seaver (1) is one of the better known heterothallic forms. As mentioned earlier, however, the linking of the colony types of brown with diffusing pigment and black with no diffusing pigment with those of (+) and (−) is particularly noteworthy. The ease with which the two "sexes" can be separated so early in colony development is advantageous as well. More and more of the discomycetous fungi are being found capable of producing apothecia in vitro (3). The present fungus should prove especially useful in studying the factors that determine each of the steps involved in the life cycle of a discomycete.

Acknowledgments

The author is indebted to Mr. R. F. Cole and Mr. A. Lutzko of this laboratory for preparation of photographic prints from negatives provided, to Dr. P. A. J. Gorin of this laboratory for investigation on the viscous liquid material, and to Dr. Olli Vaartaja, Forest Pathology Laboratory, Saskatoon, for a group of "crystal producing" cultures received in 1953.

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Explanation of Figures

Figs. 1–9. Colony characteristics and ascospore germination.

Fig. 1. Brown (+) (left) and black (−) type colonies on Difco potato dextrose agar. Inset: vertical freehand section through above colonies. Brown (+) colony on left. After 8 days' incubation at 28° C. ×ca. 0.8

Fig. 2. As for Fig. 1 but after 15 days' incubation at 28° C. ×ca. 0.8

Fig. 3. Four brown (+) and four black (−) type colonies from the eight ascospores taken from a single ascus. After 7 days' incubation at 28° C. ×ca. 0.8

Fig. 4. Same as Fig. 3 but after 21 days' incubation at 28° C. Rows of apothecia appear between the brown (+) and the black (−) type colonies. ×ca. 0.5

Fig. 5. Apothecial initials along line of junction between a brown (+) and a black (−) type colony. ×ca. 4

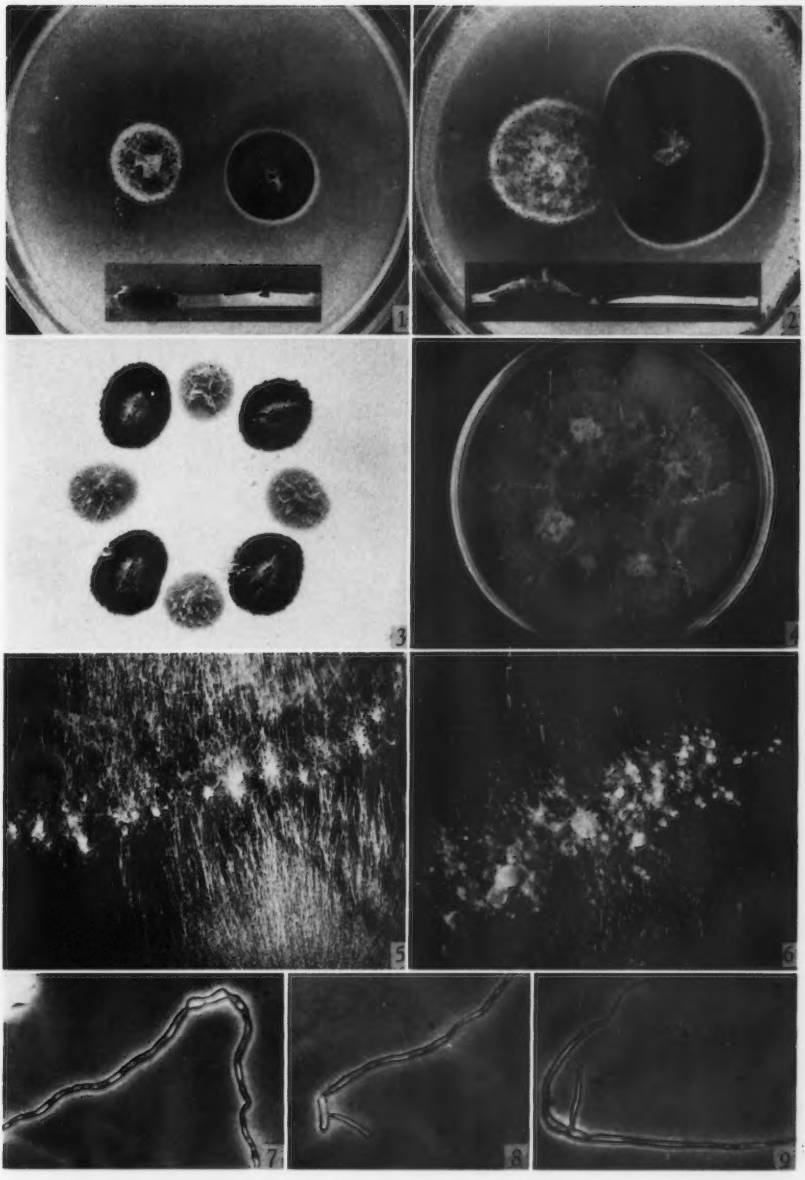
Fig. 6. As for Fig. 5 but later with mature apothecia present. ×ca. 4

Fig. 7. Germinating ascospore on agar surface. After 24 hours at room temperature. Phase ×750

Fig. 8. Germinating ascospore on agar surface. After 24 hours at room temperature. Phase ×750

Fig. 9. Germinating ascospore on agar surface. After 24 hours at room temperature. Phase ×750

PLATE I



Haskins—Can. J. Microbiol.

PLATE II

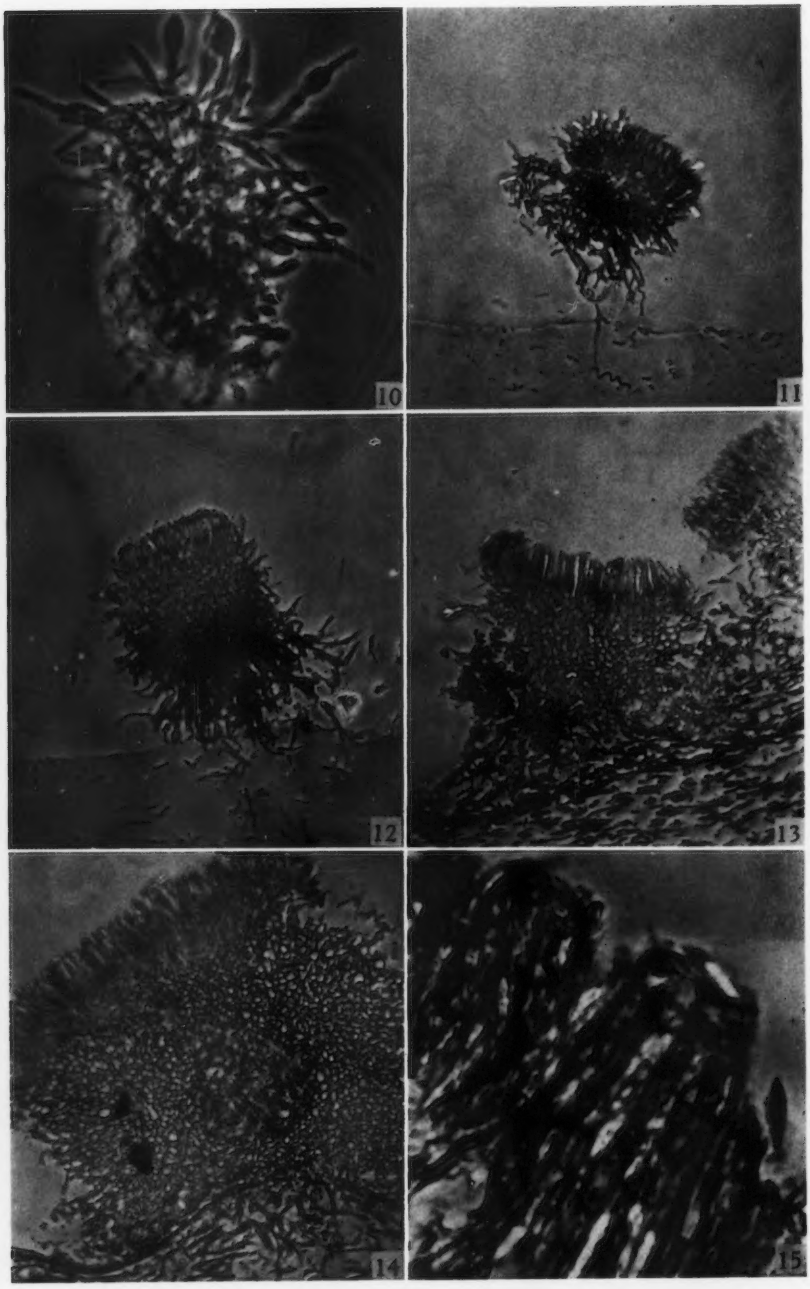


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PLATE III

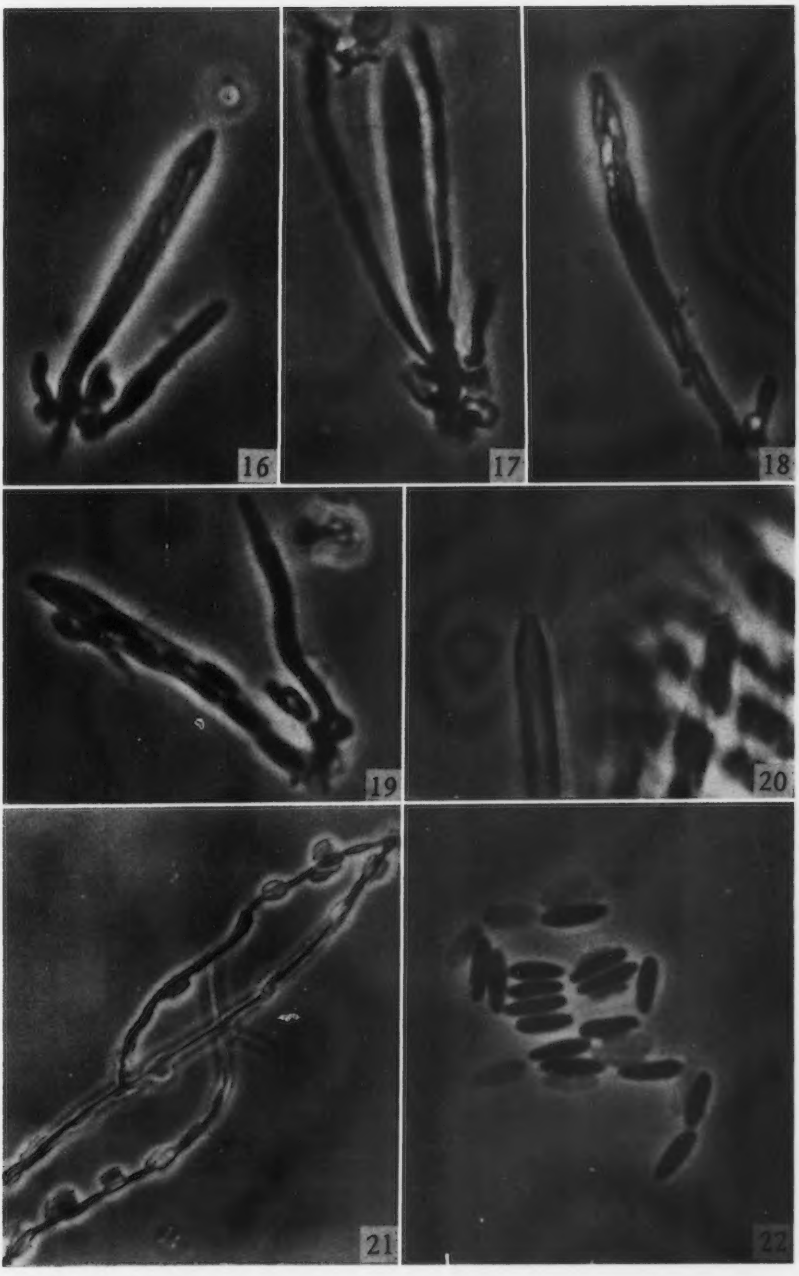

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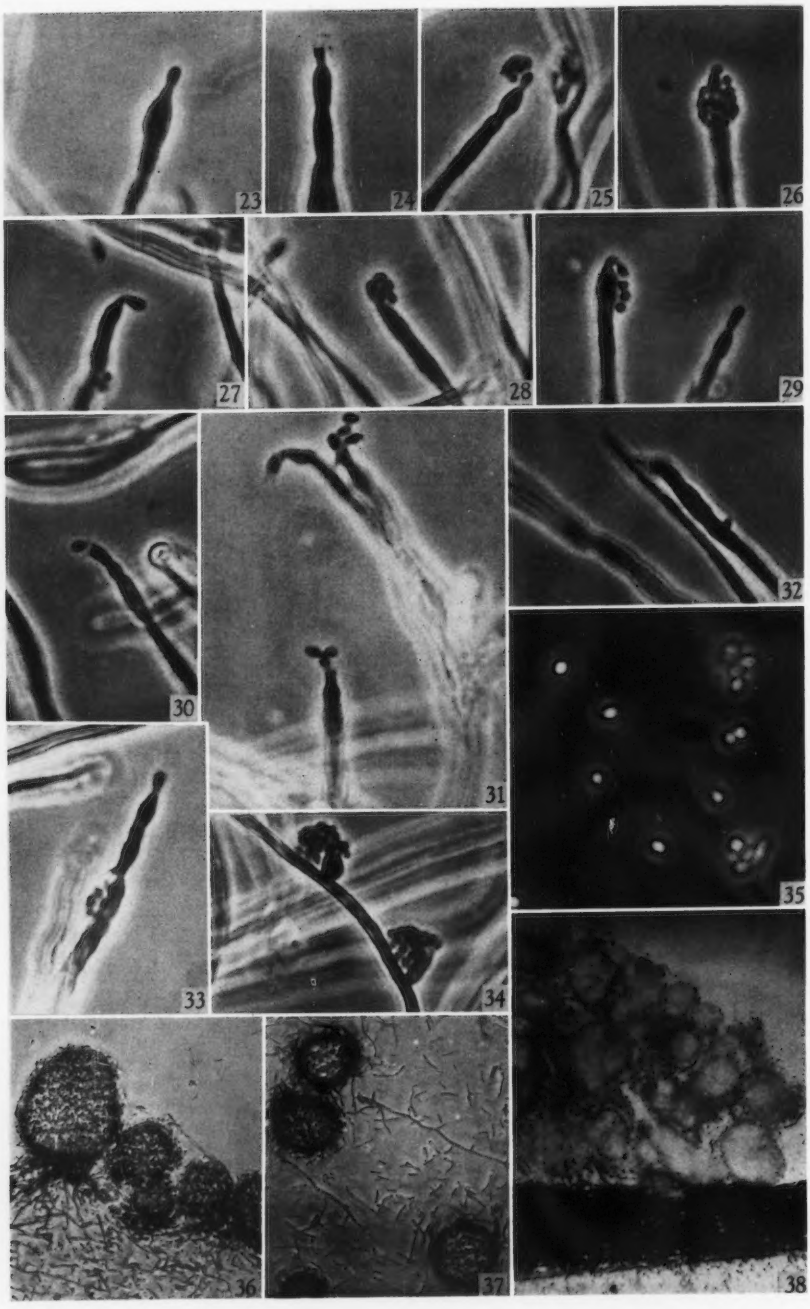
PLATE IV



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FIGS. 10–15. Apothecium development.
FIG. 10. Apothecial initial teased from surface of agar plate culture. In lactophenol. Phase ×ca. 621
FIG. 11. Young apothecium. Vertical section of paraffin-embedded portion of colony on agar. Phase ×138
FIG. 12. Apothecium. Vertical section of paraffin-embedded portion of colony on agar. Phase ×138
FIG. 13. Mature apothecium. Vertical section of paraffin-embedded portion of colony on agar. Phase ×138
FIG. 14. Mature apothecium. Vertical section of paraffin-embedded portion of colony on agar. Phase ×138
FIG. 15. Portion of hymenium of overmature apothecium. Vertical section of paraffin-embedded portion of colony on agar. Ascospores remaining undischarged have become dark-walled and septate. Phase ×1380
FIGS. 16–22. Asci, paraphyses, and ascospores.
FIG. 16. Ascus and young asci. Teased from mature apothecium. In lactophenol. Phase ×1600
FIG. 17. Ascus and paraphyses. Teased from mature apothecium. In lactophenol. Phase ×1600
FIG. 18. Ascus with ascospores. Teased from mature apothecium. In lactophenol. Phase ×1600
FIG. 19. Mature ascus with eight ascospores. Teased from mature apothecium. In lactophenol. Phase ×1600
FIG. 20. Distal end of discharged ascus showing escape orifice. In lactophenol. Phase ×1600
FIG. 21. Aerial mycelium with adhering groups of ascospores. In lactophenol. Phase ×240
FIG. 22. Discharged ascospores on agar surface. Several are already one-septate. Phase ×1600
FIGS. 23–38. Fruiting structures of black (−) strain.
FIG. 23. Initial elongated phialospore emerging from terminal phialide. In lactophenol. Phase ×1000
FIG. 24. Phialospore on terminal phialide with collar evident. In lactophenol. Phase ×1000
FIG. 25. Group of phialospores at terminal phialide. Endogenous formation of spores indicated. In lactophenol. Phase ×1000
FIG. 26. Group of phialospores clustered around phialide below its tip. In lactophenol. Phase ×1000
FIG. 27. Initial elongated phialospore emerging from terminal phialide with terminal cup curved back. In lactophenol. Phase ×1000
FIG. 28. Terminal phialide and phialospores. In lactophenol. Phase ×1000
FIG. 29. Terminal phialides. Curving back of cup discharges spores towards the phialide. In lactophenol. Phase ×1000
FIG. 30. Succession of spores in terminal phialide. In lactophenol. Phase ×1000
FIG. 31. Terminal phialides and phialospores. In lactophenol. Phase ×1000
FIG. 32. Series of "spores" forming in terminal phialide. In lactophenol. Phase ×1000
FIG. 33. Proliferation of terminal phialide from end of phialide but not through cup. In lactophenol. Phase ×1000
FIG. 34. Lateral, short phialides with phialospore clusters. In lactophenol. Phase ×1000
FIG. 35. Phialospores on agar surface. Phase ×1200
FIG. 36. Pycnidia-like bodies on surface of culture. Vertical section through paraffin-embedded portion of colony on agar. Phase ×100
FIG. 37. Subsurface pycnidia-like bodies. Section through paraffin-embedded portion of colony on agar. Phase ×100
FIG. 38. Portion of a large group of pycnidia-like bodies on surface of culture. Vertical section through paraffin-embedded portion of colony on agar. Note thin layer of highly pigmented mycelium at surface of the agar colony, typical of black (−) strains. Phase ×70

NOTES

PREPARATION OF BACTERIAL SPORE EXTRACTS BY USE OF THE EPPENBACH COLLOID MILL[1]

R. J. O'Connor, R. H. Doi, and H. Halvorson[2]

The increasing emphasis on the study of the enzymic activity of bacterial spores has necessitated the adoption of an efficient method for the preparation of active spore extracts. Although a variety of methods for the fragmentation of microbial cells has been devised (5), none of these is well suited for the production of subcellular spore preparations in large volume. Recently Garver and Epstein (3) have adapted an Eppenbach laboratory colloid mill for the disintegration of microbial cells. This report deals with the application of this technique for the rapid rupture of large amounts of bacterial spores for the production of high yields of active enzymes.

A suspension of lyophilized *Bacillus cereus* strain T spores (50 g per 500 ml phosphate buffer, 0.1 *M*, pH 6.9) was heat-shocked at 65° C for 1½ hours. The spores were then centrifuged from the heat-shock medium, washed and resuspended in 250 ml phosphate buffer, 0.1 *M*, pH 6.9, and placed in an Eppenbach colloid mill (model QV-6, Gifford-Wood Co., Hudson, New York) with 60 ml of glow beads (120–130 microns in diameter; Minnesota Mining and Manufacturing Co.) per 100 ml of suspension. The mill was operated as described by Garver and Epstein except that the mill setting (the clearance between the rotor and the stator) was 0.025 in.; temperature was maintained at 10–15° C.

Viable count was determined by plate count with nutrient agar (Difco) and incubation at 30° C for 24 hours, direct count by use of the AO Spencer Bright Line haemacytometer, protein by the method of Lowry *et al.* (7), diaphorase according to de Bernard (1), glucose dehydrogenase by the method of Strecker (9), and alanine dehydrogenase according to Goldman (4).

The rate of fragmentation is shown in Fig. 1. The viable count decreased 88% within the first 20 minutes of rupture; during this same period, the direct count decreased 65%. The discrepancy between the two counts is probably due to difficulty in distinguishing between whole viable cells and nonviable, partially fragmented spore hulls. The bulk of the spore dipicolinic acid is released within the first 10 minutes. The solubilization of enzymes generally parallels the rapid liberation of protein. The decrease in diaphorase activity was probably caused by its heat lability at temperatures above 0° C.

Table I shows the relative breakage efficiency of the colloid mill in comparison with other methods. The Waring blendor is also effective; however, the excessive foaming of the extract during rupture leads to inactivation of

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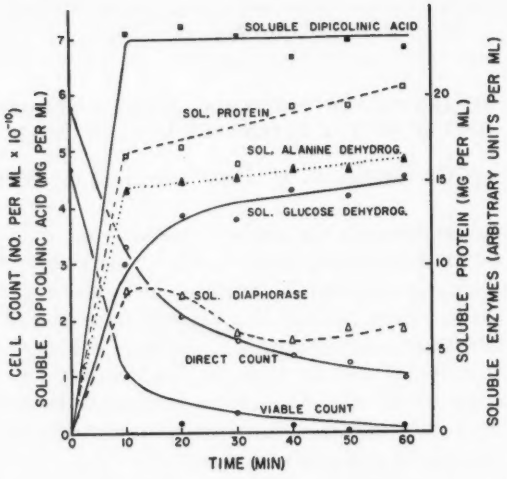

FIG. 1. Solubilization of spore components by fragmentation with the colloid mill. Experimental procedure is described in the text. Determination of spore dipicolinic acid (prior to rupture) by the method of Janssen *et al.* (6) yielded 7.34 mg per ml of spore suspension. To obtain absolute enzyme units per ml:
alanine dehydrogenase—multiply by 0.161 = μmoles DPNH oxidized per minute;
glucose dehydrogenase—multiply by 0.214 = μmoles DPN reduced per minute;
diaphorase—multiply by 0.322 = μmoles of DPNH oxidized per minute.
Final volume was 185 ml.

enzyme preparations. Although satisfactory breakage with the Mickle disintegrator has been achieved in other laboratories (2, 8), we have observed a decrease in viability of only 40% over a 60-minute period using this apparatus. The low breakage which we obtained is probably due to our relatively high concentration of beads (2.5 g per ml).

An indication of the enzyme stability during rupture from the colloid mill can be obtained by comparing the specific activities of the enzymes obtained by this and other methods. By sonic oscillation, extracts containing glucose dehydrogenase with a specific activity of 2.8 units per mg protein have been

TABLE I

Comparison of colloid mill breakage with other methods*

Time, min	Decrease in viability, %		
	Sonic oscillator (Raytheon 10 kc)	Waring blendor	Colloid mill
0	0	0	0
10	16	56	78
20	27	84	96
30	39	98	92
60	52	99	97

*The colloid mill was operated as described in the text. The spore slurries used in the sonic oscillator and the Waring blendor contained 2 g of spores and 50 g beads in 20 ml of phosphate buffer, 0.1 *M*, pH 6.9. The temperature maintained in the sonic oscillator was 8° C, that in the Waring blendor, 5–7° C.

obtained; the colloid mill yields the same enzyme at 9.9 units per mg protein. Spore alanine dehydrogenase obtained by sonic oscillation has a specific activity of 18.1 units per mg protein while the enzyme released by use of the colloid mill has an activity of 85 units per mg protein.

A striking feature of colloid mill rupture is the liberation of spore components during short periods of operation of the apparatus. Initially, there is a rapid release of dipicolinic acid paralleled by the solubilization of spore protein possessing enzymatic activity. At a period when 97% of the spore dipicolinic acid has been liberated (10 minutes), the rate of protein and enzyme solubilization falls to a comparatively low value.

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EFFECT OF NOXIVERSIN ON BACTERIAL TOXINS WHICH ALTER DERMAL REACTIVITY OF RABBITS TO EPINEPHRINE[1]

E. Neter, E. A. Gorzynski, and H. Anzai

Murray and associates (2) recently reported on a toxin-inactivating substance, noxiversin, isolated from *Penicillium cyaneo-fulvum*. This material, which differs from antibiotics produced by the same microorganism, has the extraordinary capacity of inactivating a variety of bacterial toxins, including the alpha and beta toxins of *Staphylococcus*, streptolysins S and O, haemolysins

[1]This investigation was supported by a research grant (E-658) from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, U.S.A.

of various clostridia, tetanus neurotoxin, as well as pertussis and diphtheria toxins. Rose and Martin (4) confirmed and extended these observations and ascribed this activity of noxiversin to protease. The present study was undertaken to determine whether noxiversin alters the toxic fractions of *Staphylococcus* and *Escherichia coli* responsible for altered dermal reactivity of the rabbit to epinephrine. Thomas (5) showed that the endotoxin of Gram-negative bacilli has a marked effect on the vascular system; intravenous injection results in lesions developing at the site of intradermally injected epinephrine. A study, to be reported in detail, has shown that it is the lipoid A fraction of this endotoxin which produces the epinephrine lesion (3). In additional studies it was found that a Boivin type extract obtained from a coagulase-positive strain (D) of *Staphylococcus aureus* has similar activity (1).

Noxiversin was kindly made available in the dried form by Dr. J. W. Stevenson of McGill University; 1 mg of this preparation neutralizes 1283 Burnet units of staphylococcal alpha haemolysin. Lipoid A was obtained from Professor Otto Westphal, Freiburg, Germany, and the staphylococcal extract from Difco Laboratories, through the courtesy of Dr. C. W. Christensen. Appropriate dilutions of the toxins in phosphate buffer of pH 7.3 were used to dissolve noxiversin. The mixtures were incubated for 1 hour in a water bath at 37° C, and then injected intravenously into white female rabbits weighing between 1.6 and 2.5 kg. Immediately thereafter epinephrine in aqueous solution and in oil was injected intradermally at different sites of the shaved abdominal skin and, for control purposes, saline solution was injected at another site. Reactions were observed after 18 hours. The results of these experiments together with appropriate controls are shown in Table I.

TABLE I

Effect of noxiversin on bacterial toxins causing altered dermal reactivity of rabbits to epinephrine

Toxins and amounts, μg/kg	Noxiversin, mg/kg	Number of rabbits	
		With positive epinephrine reactions	Tested
Staphylococcal extract			
28	2	2	2
10	3–3.3	4	4
10	1.5	2	2
20	0	103	112
10	0	9	10
E. coli lipoid A			
20	1.7–1.9	6	8
20	0	22	37

Perusal of Table I reveals that noxiversin in the amounts used did not abolish the toxic activity of lipoid A and of the staphylococcal extract which cause altered dermal reactivity to epinephrine. It should be emphasized that, on a weight basis, from 70 to 330 times as much of noxiversin than of bacterial

toxins were employed. It is conceivable that neutralization would have been observed had larger amounts of noxiversin been used. On the other hand, if, as suggested by Rose and Martin (4), noxiversin's activity is proteolytic in nature, then it is to be expected that this protease does not affect either the lipoid A component of endotoxin from Gram-negative bacilli or the heat-stable staphylococcal extract.

In summary, noxiversin, in the amounts used in these studies, did not abolish the toxic activity of two bacterial products which result in altered reactivity to epinephrine in the rabbit.

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A STUDY OF BACTERIOLOGICAL MEDIA: THE EXAMINATION OF PEPTIDES IN CASAMIN E[1]

A. F. S. A. HABEEB[2]

The separation and identification of peptides in culture media is essential as a preliminary to the study of growth-stimulating factors. The preliminary separation of peptides in "Oxoid" bacteriological peptone (1, 2) was carried out using columns of silica gel, acid alumina, and charcoal. The use of paper electrophoresis at pH 5.8 followed by paper chromatography for the fractionation of peptides in "Difco" bacto-casitone was described (4). This note describes the use of paper electrophoresis at two pH values: pH 5.8 and then pH 2.4, together with paper chromatography to separate and identify the peptides in casamin E.

The apparatus for paper electrophoresis (4) which is a modification of that by Kunkel and Tiselius (7) was used. Three batches were subjected to the

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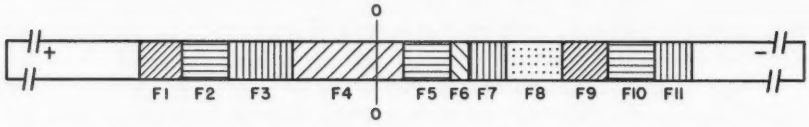


FIG. 1. Electrophoretic pattern of casamin batch B at pH 5.8. 0—0 line of application. A, B, C represent batches, + the anode, − the cathode.

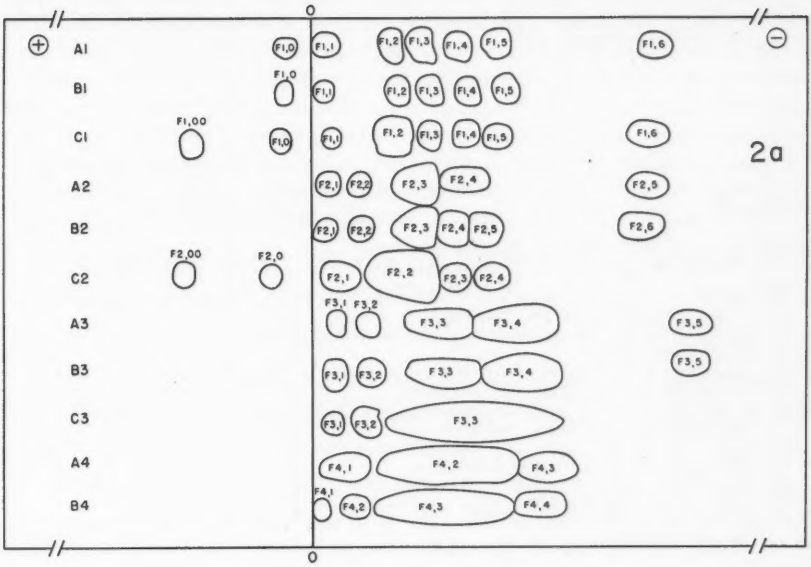
electrophoretic separation at pH 5.8 as previously described (4). Batches A and B gave 11 bands while batch C gave 8 bands. Figure 1 shows the pattern obtained for batch B. Each band was eluted and an aliquot was subjected to electrophoresis at pH 4.3, pH 3, and pH 2.4 to find out the most suitable pH for their separation. It was found that pH 2.4 (1.2 *N* acetic acid) was optimum for the electrophoretic fractionation of the bands (Fig. 2, *a* and *b*). The spots obtained were compact; some showed elongation which may be due to small differences in the electrophoretic mobilities of their components. Batches A, B, and C thus showed 44, 38, and 33 fractions respectively. The fractions were chromatographed on Whatman No. 1 paper with butanol – acetic acid – water system as developer. Table I summarizes the results obtained from the three batches of casamin. Each subfraction was examined (4) for its amino acid content and for the N-terminal amino acid(s) of its constituent peptide(s). Results showed variation in the amino acid content of subfractions among the three batches. The acidic subfractions were rich in aspartic and glutamic acids and poor in the basic amino acids; the reverse was true for the basic subfractions. Several of the subfractions showed more than one N-terminal amino acid which is evidence to their inhomogeneity. Some fractions of the three batches that showed similar electrophoretic mobilities (Fig. 2, *a* and *b*) gave rise to subfractions that varied in their amino acid content and the N-terminal residue.

TABLE I

Analysis of casamin E

	Number of bands			Number of fractions			Number of subfractions		
	A	B	C	A	B	C	A	B	C
Acidic	4	4	3	20	21	17	52	58	50
Neutral	1	1	1	2	2	1	17	17	9
Basic	6	6	4	22	15	15	69	44	43
Total	11	11	8	44	38	33	138	119	102

Casamin E is a pancreatic digest of casein; it has been found by Keller *et al.* (6) that the proteolytic components of bovine pancreatic juice are composed of trypsin, chymotrypsin, and two carboxypeptidases. The action of these enzymes on casein thus results in a very complex mixture of peptides and any slight variation in the conditions of the enzymatic digestion will result in variation in the peptides produced. However by the use of paper electrophoresis at pH 5.8 then at pH 2.4 followed by paper chromatography it was possible



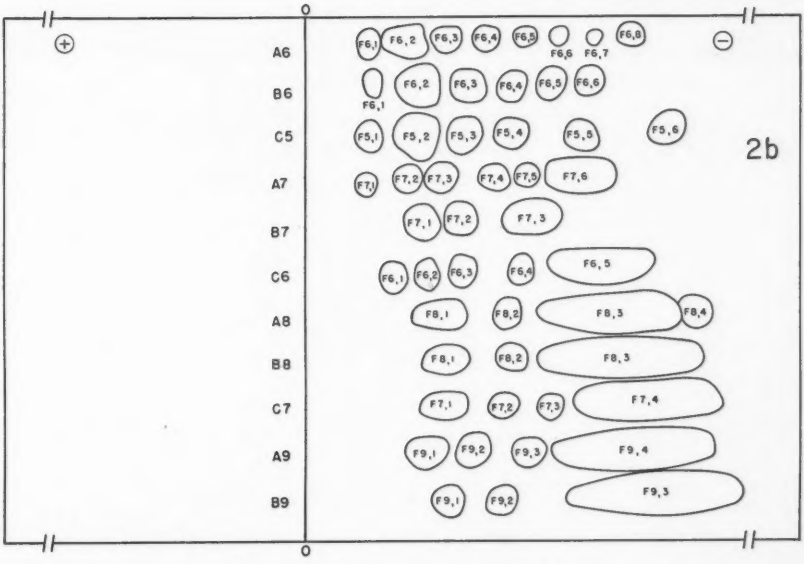


FIG. 2*a*. Paper electrophoresis of the acidic bands at pH 2.4. 0—0 line of application; number of band follows A, B, and C.

FIG. 2*b*. Paper electrophoresis of the basic bands at pH 2.4. 0—0 line of application; number of band follows A, B, and C.

to resolve the peptide content of casamin E into 102–138 subfractions. The number of peptides present is probably much greater which results in difficulty of obtaining complete resolution into single peptides. The pancreatic digestion of proteins results in non-specific cleavage of the peptide bonds, thereby variation occurs in the amino acids and peptides among different batches from the same manufacturer (3, 5) and from different manufacturers (3, 5).

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Contents

Page

Joyce C. Lewin and Ralph A. Lewin—Auxotrophy and heterotrophy in marine littoral diatoms 127

D. G. Lundgren and G. Beskid—Isolation and investigation of induced asporogenic mutants 135

William A. Corpe—The extracellular polysaccharide of gelatinous strains of *Chromobacterium violaceum* 153

S. N. Sehgal and N. E. Gibbons—Effect of some metal ions on the growth of *Halobacterium cutirubrum* 165

Robert-A. Lachance—The vitamin requirements of *Corynebacterium sepedonicum* (Spiek. & Kott.) Skapt. & Burkh. 171

R. C. French and R. E. Armstrong—Thermal inactivation of MEF1 poliovirus . 175

J. Robinson, F. S. Thatcher, and Jeannine Montford—Studies with staphylococcal toxins. V. Possible identification of alpha hemolysin with a proteolytic enzyme 183

J. Robinson, F. S. Thatcher, and Jeannine Montford—Studies with staphyloccocal toxins. VI. Some properties of a non-hemolytic dermonecrotic toxin produced by some strains of *Staphylococcus aureus* 195

Tadayo Hashimoto, Samuel H. Black, and Philipp Gerhardt—Development of fine structure, thermostability, and dipicolinate during sporogenesis in a bacillus 203

Samuel H. Black, Tadayo Hashimoto, and Philipp Gerhardt—Calcium reversal of the heat susceptibility and dipicolinate deficiency of spores formed "endotrophically" in water 213

R. H. Haskins—A heterothallic, inoperculate discomycete: morphology and development 225

Notes:

R. J. O'Connor, R. H. Doi, and H. Halvorson—Preparation of bacterial spore extracts by use of the Eppenbach colloid mill 233

E. Neter, E. A. Gorzynski, and H. Anzai—Effect of noxiversin on bacterial toxins which alter dermal reactivity of rabbits to epinephrine 235

A. F. S. A. Habeeb—A study of bacteriological media: the examination of peptides in casamin E 237